


AB






EUROPEAN PATENT APPLICATION

 Application number : **94304432.1**


 Int. Cl.⁵ : **C12N 15/63, A61K 31/00**


 Date of filing : **20.06.94**

 Priority : **21.06.93 US 81610**
18.05.94 US 246990

 Inventor : **Yang, Na Nora**
2750 Wood Wind Way
Indianapolis, Indiana 46268 (US)


 Date of publication of application :
21.12.94 Bulletin 94/51

 Representative : **Hudson, Christopher Mark et al**
Lilly Industries Limited
European Patent Operations
Erl Wood Manor
Windlesham Surrey GU20 6PH (GB)

 Designated Contracting States :
AT BE CH DE DK ES FR GB GR IE IT LI LU NL
PT SE

 Applicant : **ELI LILLY AND COMPANY**
Lilly Corporate Center
Indianapolis Indiana 46285 (US)

 **Materials and methods for screening anti-osteoporosis agents.**

 The present invention relates to methods for the identification of therapeutic agents for the treatment of osteoporosis and serum lipid lowering agents. The invention relates to isolating cloning, and using nucleic acids from the promoter regions of transforming growth factor β genes comprising novel regulatory elements designated "raloxifene responsive elements". The invention also encompasses eukaryotic cells containing such raloxifene responsive elements operably linked to reporter genes such that the raloxifene responsive elements modulate the transcription of the reporter genes. The invention provides methods for identifying anti-osteoporosis agents that induce transcription of genes operably linked to such raloxifene responsive elements without inducing deleterious or undesirable side effects associated with current anti-osteoporosis therapy regimens.

of breast tumors, but it also acts as an agonist in its ability to reduce the amount of serum cholesterol in both healthy women and women with breast cancer. Love *et al.*, *Annals Int. Med.*, **115**, 860-864 (1991). Tamoxifen also act to increase bone density in breast cancer patients. Love *et al.*, *N. Eng. J. Med.*, **326**, 852-856 (1991). At least one study has suggested that the increases in bone density possible with tamoxifen appear to be restricted to the lumbar spine, with bone loss being reported in the radius in some breast cancer patients treated with tamoxifen. Furthermore, tamoxifen treatment has also been suggested to contribute to weight gain among post-menopausal women. Love *et al.*, *Ann. Int. Med.*, *ibid.*

Improved anti-osteoporotics that achieve increases in bone density without causing negative side effects are clearly needed. Unfortunately, no method currently exists for rapidly and efficiently screening large numbers of compounds to identify those that display the desired anti-osteoporotic effects. Because this screening process comprises the most time-consuming and expensive step in identifying improved anti-osteoporotic compounds, development of a rapid method for testing large numbers of compounds to identify those that are likely to possess anti-osteoporotic effect is highly desirable.

It is well established that estrogens exert their effects by first binding to an estrogen receptor, and then the estrogen/estrogen receptor complex binding to DNA. The hormone/receptor complex modulates gene expression via this DNA binding. Kumar, *Cell*, **55**, 145-156 (1988). Antiestrogens also bind to estrogen receptors. Although these antiestrogen/receptor complexes bind to DNA they generally fail to modulate gene expression. Both estradiol/estrogen receptor complexes and hydroxytamoxifen/estrogen receptor complexes bind *in vitro* to DNA binding domains called estrogen responsive elements. Kumar, *Cell*, *ibid.*

The conformation of the ligand/receptor complex is a matter of some debate. However, recent studies have suggested a conformational difference between estrogen receptor bound to estradiol and the same estrogen receptor bound to 4-hydroxytamoxifen or ICI 164,384. Klinge *et al.*, *J. Ster. Biochem. Mol. Biol.*, **43**, 249-262 (1992).

In an effort to rationally address the problem of developing improved anti-osteoporotic agents, researchers have investigated proteins known to play a role in bone maintenance. One protein known to influence bone remodelling and bone turnover is transforming growth factor β (TGF β). Although commonly referred to as a single compound, TGF β is actually a family of molecules that now is known to include at least three isoforms: TGF β -1, TGF β -2 and TGF β -3. See Arrick *et al.*, *Canc. Res.*, **50**, 299-303 (1990). The present inventor has noted that ovariectomy induces a significant decrease in TGF β -3 in rat bone (data collected by present inventor is unpublished); others have noted the same type of correlation with respect to levels of TGF β (isoform not specified). See Finkelman *et al.*, *Proc. Nat'l Acad. Sci. USA*, **89**, 12190-12193 (1992). Further, the present inventor has noted that administration of raloxifene, an antiestrogen, to ovariectomized rats restores TGF β -3 concentrations to levels equal to or higher than that found in control animals. The direct correlation between TGF β -3 levels and circulating levels of estrogen or antiestrogen, and the finding that TGF β (isoform not specified) plays a significant role in bone remodelling and turnover, suggest that osteoporosis may result from reduced expression of TGF β -3 *in vivo*. See Noda *et al.*, *Endocrin.*, **12**, 2991-2994 (1989).

The hypothesis that reduced levels of TGF β -3 may allow bone loss is undermined by the findings that TGF β has been isolated from a large number of sources and exhibits widely divergent effects. For example, it inhibits the growth of mesenchymal cells and epithelial cells, it induces biosynthesis of proteoglycans, fibronectins, and plasminogen activator, and is chemotactic for fibroblasts, macrophages, and smooth muscle cells. See, Flaumenhaft *et al.*, *J. Cell. Bio.*, **120**(4), 995-1002 (1993).

Furthermore, antiestrogens such as tamoxifen or toremifene induce human fetal fibroblasts to secrete TGF β (without reference to isoform) in the absence of estrogen receptor Colletta *et al.*, *Br. J. Cancer*, **62**, 405-409 (1990). TGF β has been found to stimulate osteoblastic bone formation and to inhibit osteoclast formation and osteoclast activities. Mundy, "Clinical Application of TGF β ", *Ciba Foundation Symposium No. 157*, 137-151, Wiley, Chichester. TGF β repressed division of one human endometrial cancer cell line (Hec-Fawa), but was shown to be mitogenic with respect to another such cell line (HEC-50) Murphy *et al.*, *J. Ster. Biochem. Molec. Bio.*, **41**, 309-314 (1992).

Three months of antiestrogen treatment with tamoxifen has been correlated with induction of extracellular TGF β -1 in breast cancer biopsies. Butta *et al.*, *Cancer Res.*, **52**, 4261-4264 (1992). Decreased concentrations of TGF β -1 mRNA were found in one human endometrial cancer cell line (HEC-50) grown in media containing 1% cFBS (twice charcoal stripped FBS) when such cells were exposed to either estradiol or certain antiestrogens. Gong *et al.*, *Canc. Res.*, **52**, 1704-1709 (1992).

TGF β -2 mRNA is expressed by the T-47D and MDA-MB-231 cell lines. Treatment of these cell lines with estradiol reduced TGF β -2 mRNA expression, but tamoxifen did not exhibit the same effect. TGF β -3 induces mitogenesis, collagen synthesis, and alkaline phosphatase activity in osteoblast enriched bone cell cultures at a three to five fold higher rate than TGF β -1. Arrick *et al.*, *Canc. Res.*, **50**, 299-303 (1990).

A general review of the properties of TGF β are described in Sporn *et al.*, *J. Cell Bio.*, **105**, 1039-1045 (1987);

Figure 12 represents the relative level of induction of reporter gene expression in MG63 cells transfected with TGF β -3 promoter/CAT expression constructs and exposed to various concentrations of the compounds set forth in Example X. Overall, compound 177366 is the most potent inducer, while 98005 shows no induction.

Figure 13 depicts the portions of the TGF β -3 promoter used to identify the 41 base pair raloxifene responsive element, and depicts the relative induction of reporter gene expression by raloxifene in cells transformed with plasmids comprising the indicated portion of the TGF β -3 promoter sequence operably linked to a reporter gene. Although the fold induction achievable with the pB-301 construct is highest, presence of the raloxifene responsive element (base pairs +35 - +75) is clearly essential for any significant induction of transcription by raloxifene in these cells.

Figure 14 depicts an analysis of the TGF β -3 promoter. The major transcriptional start site and a CCCTC-motif are depicted as described in Example XI.

Figure 15 depicts the relative expression of reporter gene in Hep2 cells transfected with an expression construct comprising the LDLR promoter and the luciferase gene in the presence of estrogen receptor and various concentrations of estradiol and raloxifene. Generally, raloxifene is the more potent inducer.

Figure 16 depicts the relative expression of reporter gene in Hep2 cells transfected with an expression construct comprising the LDLR promoter and the luciferase gene in the presence of various concentrations of estradiol and raloxifene and in the absence of estrogen receptor. No induction is exhibited by either compound at concentrations at or below 10^{-6} M. High concentrations of raloxifene induce expression somewhat, suggesting an alternate, non-ER dependent induction mechanism at such concentrations.

Figure 17 is a flow diagram showing an example of a sequence of steps that can be carried out according to the teachings of the present invention to evaluate compounds with respect to their ability to induce transcription of reporter genes operably linked to the regulatory control element described herein. It is expected that a correlation will exist between compounds showing the induction profiles described in Example XIII and the ability of such compounds to act as anti-osteoporosis drugs *in vivo*.

According to the present invention, there is provided novel and efficient methods for screening chemical compounds to determine whether these compounds are capable of modulating steroid hormone-responsive gene expression from a promoter comprising a raloxifene responsive element. There is also provided nucleic acids consisting essentially of a nucleotide sequence comprising a raloxifene responsive element isolated from the promoter region of a TGF β gene, and eukaryotic cells transfected therewith. There is also provided a recombinant expression construct comprising the raloxifene responsive element operably linked to a reporter gene. There is also provided a method for inducing bone formation, a method for treating osteoporosis, and a method for treating bone fractures which comprise administering a compound that, when bound to an estrogen receptor, potentially induces transcription from a raloxifene responsive element of a promoter region of a TGF β gene.

The present invention relates to novel and efficient methods of screening chemical compounds to determine whether those compounds are capable of modulating steroid hormone-responsive gene expression from a mammalian promoter comprising a raloxifene responsive element as discovered and described herein. The invention comprises nucleic acids consisting essentially of the nucleotide sequence of a mammalian promoter comprising such a raloxifene responsive element. In a preferred embodiment, the promoter comprising the raloxifene responsive element is derived from the promoter region of the gene for human TGF β -3 or TGF β -2.

The invention further comprises recombinant eukaryotic expression constructs comprising a promoter having a raloxifene responsive element that is operably linked to a reporter gene. In preferred embodiments, the reporter gene is the chloramphenicol acetyltransferase gene or the luciferase gene. Particularly, preferred is the luciferase gene. Cells transfected with such eukaryotic expression constructs, that are capable of expressing the reporter gene when such cells are exposed to raloxifene or other anti-estrogenic compounds, are also provided by the invention.

The present invention further comprises a method for inducing bone formation which comprises administering an effective amount of a compound that, when bound to an estrogen receptor, potentially induces transcription from a raloxifene responsive element of a promoter region of a TGF β -3 gene. The present invention also includes a method for treating osteoporosis which comprises administering an effective amount of a compound that, when bound to an estrogen receptor, potentially induces transcription from a raloxifene responsive element of a promoter region of a TGF β -3 gene. The present invention also provides a method for treating bone fractures which comprises administering an effective amount of a compound that, when bound to an estrogen receptor, potentially induces transcription from a raloxifene responsive element of a promoter region of a TGF β -3 gene.

In the first aspect, the invention provides a nucleic acid consisting essentially of a nucleotide sequence comprising a raloxifene responsive element, where the element is isolated from the promoter region of a mammalian, preferably human, transforming growth factor β gene. In preferred embodiments, the transforming

are embodiments wherein the transforming growth factor β gene is the human TGF β -2 gene or the human TGF β -3 gene. In other preferred embodiments, the reporter gene is the chloramphenicol acetyltransferase gene or the luciferase gene. In further preferred embodiments of this aspect of the invention, the raloxifene responsive promoter sequences consisting essentially of the promoter sequences of the TGF β -3 gene comprising the plasmids pB-301 (containing TGF β -3 promoter sequences from positions -301 to +110); pB-221 (containing TGF β -3 promoter sequences from positions -221 to +110); pB-91 (containing TGF β -3 promoter sequences from positions -91 to +110); pB-60 (containing TGF β -3 promoter sequences from positions -60 to +110); pB-47 (containing TGF β -3 promoter sequences from positions -47 to +110) and pB-38 (containing TGF β -3 promoter sequences from positions -38 to +110), as further described herein and operably linked to a reporter gene.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

This specification contains a number of abbreviations. As used herein, "TGF β " shall mean transforming growth factor β (without reference to isoform). TGF β -1, TGF β -2 and TGF β -3 shall have the meanings well-established in this art, i.e., to represent the three known isoforms of transforming growth factors β genes. As used herein, the abbreviation "CAT" shall be taken to mean chloramphenicol acetyl CoA transferase. "Estradiol" is an estrogen and, at times, is abbreviated herein as E2. As used herein, the abbreviation "ER" shall mean an estrogen receptor protein.

The term "raloxifene responsive element" as used herein refers to nucleotide sequences of the nucleic acid comprising a mammalian promoter region of the TGF β gene that are capable of inducing transcription of any structural gene to which the raloxifene responsive element is operably linked in host cells that are exposed to raloxifene and estrogen receptor proteins. Raloxifene responsive elements include, but are not limited to the nucleotide sequences comprising the TGF β promoter sequences of the plasmids pB-301 (containing TGF β -3 promoter sequences from positions -301 to +110); pB-221 (containing TGF β -3 promoter sequences from positions -221 to +110); pB-91 (containing TGF β -3 promoter sequences from positions -91 to +110); pB-60 (containing TGF β -3 promoter sequences from positions -60 to +110); pB-47 (containing TGF β -3 promoter sequences from positions -47 to +110) and pB-38 (containing TGF β -3 promoter sequences from positions -38 to +110), as further described herein, and nucleic acids having substantially the same biological activity as those nucleic acids. This definition is intended to encompass natural allelic variations in the promoter regions of the TGF β genes. Isolated raloxifene responsive elements of the present invention may be derived from TGF β promoters of any mammalian species of origin, but are preferably of human origin.

As used herein, anti-estrogens will be taken to include full and partial antagonists of estrogen. All estradiols used in the Examples described herein are 17 β -estradiol.

The term "effective amount" represents an amount of a compound that, when bound to an estrogen receptor, is capable of inducing transcription from a raloxifene responsive element when administered to a mammal. The particular dose of compound administered will be determined by the particular circumstances surrounding the case, including the compound administered, the route of administration, the particular condition being treated, and similar considerations.

The term "potently" represents a compound that, when bound to an estrogen receptor, induces transcription from a raloxifene responsive element at a minimum effective concentration (MEC) of less than or equal to 10nM (1×10^{-8} M), when the compound is tested in the *in vitro* assay described herein. See Examples V, VI, X, and XIV.

All portions of promoter sequences are identified in terms of their distance, in number of nucleotides, from the major transcriptional start site of the gene, taking this start site to be +1 as shown in Figures 1-3. A negative sign (-) preceding the number indicates the nucleotide is 5' to the start site, a positive sign (+) preceding the number indicates the nucleotide is 3' to the start site. The sequences are also identified by the numbering indicated in SEQ ID NOS:1-3, and are specifically correlated with numbering of Figures 1-3.

DNA that encodes the raloxifene responsive elements of the present invention may be obtained, in view of the instant disclosure, by chemical synthesis, by *in vitro* amplification [including but not limited to the polymerase chain reaction (PCR)], or by combinations of these procedures from naturally-occurring sources, such as cultures of mammalian cells, genomic DNA from such cells, or libraries of such DNA.

The raloxifene responsive elements may be advantageously operably linked to reporter genes and used to either transiently or stably transform appropriate host cells through the use of appropriate vectors, constructs, and means well known in the art, such as DNA mediated gene transfer means including but not limited to transfection, electroporation, and virally-mediated infection. The term "recombinant expression construct" as used herein is intended to mean DNA constructs capable of directing the expression of reporter genes to which the raloxifene responsive elements of the invention are operably linked.

DNA regions are operably linked when they are functionally related to each other. For example, a promoter

Biol., 2, 1044-1051 (1982)) operably linked to promoter sequences from the TGF β -1, TGF β -2 and TGF β -3 genes were obtained from A. Roberts, National Institutes of Health, Laboratory of Chemoprevention (NIH/NCI, Bethesda, MD). These plasmids were designated pHG12 (TGF β -1), pTGF-1 (TGF β -2) and pB-499 (TGF β -3), respectively. The sequences for each of these promoters can be found in Kim *et al.*, *J. Biol. Chem.*, 264, 402-408 (1989) (TGF β -1); Noma *et al.*, *Growth Factor*, 4, 247-255 (1991) (TGF β -2); and Lafyatis *et al.*, *J. Biol. Chem.*, 265, 19128-19136 (1990) (TGF β -3). The promoter sequence of the TGF β -1 gene has been submitted to GenBank/EMBL Data Bank under accession number J04431. The TGF β -1, TGF β -2 and TGF β -3 promoter sequences are shown in Figures 1, 2 and 3, respectively, and as SEQ ID NOS: 1, 2 and 3, respectively.

Alternatively, CAT-containing reporter plasmids operably linked to each of the TGF β promoter sequences can be produced by subcloning each TGF β promoter into a commercially-available CAT construct, for example, pCAT-Basic (Promega, Madison, WI), using conventional cloning techniques. See Sambrook, Fritsch, and Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Press, Cold Spring Harbor, New York (hereinafter Sambrook *et al.*).

In order to identify region(s) of the TGF β -3 gene promoter responsive to the antiestrogen raloxifene, CAT reporter gene expression directed by constructs containing partial sequences of the TGF β -3 gene promoter were analyzed. Six TGF β -3 promoter deletion/CAT reporter constructs were obtained from A. Roberts. Plasmid designations and the extent of the promoter region contained in each of these plasmids are set forth below:

pB-301	-301 to +110	(corresponding to 1896 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-221	-221 to +110	(corresponding to 1976 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-91	-91 to +110	(corresponding to 2106 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-60	-60 to +110	(corresponding to 2137 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-47	-47 to +110	(corresponding to 2150 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-38	-38 to +110	(corresponding to 2159 to 2306 shown in Figure 3 and SEQ ID NO:3)

Two additional TGF β -3 promoter deletion constructs were constructed as described below. The first of these consisted of human TGF β -3 promoter region sequences corresponding to positions -38 to +75 in the promoter sequence, corresponding to 2159 to 2271 shown in Figure 3 and SEQ ID NO:3 (see Lafyatis *et al.*, *ibid.*). The second promoter deletion construct consisted of human TGF β -3 promoter region sequences corresponding to positions -38 to +35 in the promoter sequence, corresponding to 2159 to 2231 shown in Figure 3 and SEQ ID NO:3. The well-established practice in this art is to identify all promoter sequences identified with respect to the distance from the transcription start site.

These plasmids were generated as follows. Oligonucleotides corresponding to the extent of the TGF β -3 promoter sequence desired in each plasmid were synthesized using a DNA/RNA synthesizer (Model 394, Applied Biosystems Inc., Foster City, CA) under β -cyanoethyl phosphoramidite synthesis conditions specified by the manufacturer. Complementary pairs of oligonucleotides for each plasmid construct were synthesized, purified, mixed, and allowed to anneal to form double-stranded DNA corresponding to the appropriate TGF β -3 promoter sequences using conventional methods (Sambrook *et al.*, *ibid.*). HindIII and XbaI restriction enzyme recognition sites were synthesized as the appropriate overhanging ends at the 5' and 3' ends of the sequences, respectively. Double-stranded promoter sequences were then ligated into the HindIII/XbaI-digested CAT reporter plasmid pB-301 and propagated in bacteria under standard conditions. The reporter plasmid produced in this way that contained the -38 to +75 region of the TGF β -3 promoter was termed pTGF β +75CAT, and the plasmid that contained the -38 to +35 region of the TGF β -3 promoter was termed pTGF β +35CAT. These plasmids were used in CAT assays as described below in Example V.

B. Luciferase reporter plasmids containing TGF β -3 promoter deletion constructs, including control containing no portion of the promoter region: pTGF β -301LUC, pTGF β -38LUC, pTGF β +75LUC, pTGF β +35LUC and pLUC

Four plasmids were constructed containing the luciferase gene (REF) expressed under the transcriptional control of TGF β -3 promoter sequences and varying deletion derivatives thereof. The plasmid pTGF-301LUC was made by digesting pB-301 with HindIII and thereafter the ends of the HindIII digestion-generated overhang were blunted by treatment with the Klenow fragment of bacterial DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN). XbaI digestion was then performed to liberate the portion of the TGF β -3 promoter correspond-

5' -GATCTCTCCAGTCGCGA-3'
(SEQ ID NO:7).

5 The resulting vector was designated pSv2-H NXB because it contained a *Bam*HI site, an *Nru*I site, an *Xho*I site and a *Bgl*II site. The *Hind*III-*Bgl*II fragment of plasmid pAlc4(NRRL B-18783), which contains the firefly luciferase gene (REF), was then ligated into the *Hind*III-*Bgl*II site of plasmid pSv2-HNXB.

The 1546 base pair fragment described above was isolated and cloned into the vector pSv2 containing firefly luciferase reporter gene that had been restriction enzyme digested to completion with *Nde*I and partially with *Hind*III. The resulting vector, pDLRLUC10 contains the human LDL receptor promoter directing expression of the firefly luciferase gene, an ampicillin resistance marker and an origin of replication.

EXAMPLE II

15 Human Estrogen Receptor Expression Plasmids

The estrogen receptor-containing mammalian expression constructs pCMVER and pRSV-ER were obtained from B.S. Katzenellenbogen, Department of Physiology and Biophysics, University of Illinois (Urbana-Champaign, IL). See Reese and Katzenellenbogen, *J. Biol. Chem.*, **266**, 10880-10887 (1990). These plasmids were used in expression assays as described below, for example, in Example VII.

EXAMPLE III

Construction of An Estrogen Responsive Element/Luciferase Gene-Containing Plasmid

25 Complementary oligonucleotides corresponding to the estrogen-responsive element (ERE) from the *Xenopus laevis* vitellogenin Az gene promoter, corresponding to positions -341 to -310 [(Metzger *et al.*, *Nature*, **334**, 31-36 (1988))] were designed, synthesized, and annealed to form a double-stranded region that is an estrogen responsive element essentially as described in Example I. A sequence comprising an *Xho*I restriction enzyme recognition site was synthesized to be flanking the ERE sequences, the element having the following nucleotide sequence (shown as SEQ ID NOS:8 and 9, respectively):

5' -TCG-AGA-AAA-GTC-AGG-TCA-CAG-TGA-CCT-GAT-CAA-AC-3'
35 3' -CT-TTT-CAG-TCC-AGT-GTC-ACT-GGA-CTA-GTT-TGA-GCT-5'

The double-stranded ERE was subcloned into *Xho*I-digested pGL12-Basic (Promega), whereby the luciferase gene was placed under the transcriptional influence of the ERE. This plasmid was designated pGL2ERELUC and used in further experiments as described below (Example VI).

EXAMPLE IV

DNA Transfection

45 A. Cell culture

Mammalian cells were cultured in media (termed 3:1 media) consisting of Dulbecco's modified Eagle's medium and F12 media (mixed in a ratio of 3:1, obtained from GIBCO, Grand Island, NY), without phenol red, containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were passaged at four day intervals. One day prior to transfection, cells were trypsinized by incubating them with 1mL of a solution of 0.05% trypsin/5.3mM tetrasodium ethylenediamine tetraacetate (GIBCO) for 5min at room temperature, and then seeded in 3:1 media containing 10% charcoal-stripped FBS (csFBS; Hyclone) at densities of one million cells per 10cm culture dish.

55 B. Transient co-transfection of TGF β constructs and human ER constructs

Co-transfection experiments were performed in MG63 (human osteosarcoma) cells, using the ProFection Mammalian Transfection System (Promega). Ten μ g of TGF β promoter-containing reporter plasmid DNA and

night incubation by aspirating the media from the culture dishes, followed by rinsing each dish twice with D-PBS. For each co-transfected cell line, the buffer was replaced with 10 ml fresh medium containing 10% csFBS and one of the following compositions:

- a. 10 μ l ethanol (hormone vehicle) = control;
- b. 10 μ l 17 β -estradiol (Sigma) ("estradiol") at a concentration of 10^{-4} M in ethanol;
- c. 10 μ l raloxifene (Eli Lilly Laboratories) at a concentration of 10^{-4} M in ethanol;
- d. 10 μ l Tamoxifen (Sigma) at a concentration of 10^{-4} M in ethanol.

After 24h, incubation with these hormonal preparations (or the vehicle control), cells were washed twice with D-PBS. The cells were then scraped from the culture dishes using a rubber policeman and 1 ml of D-PBS. Cells were collected by centrifugation at 8,000 rpm for two minutes in a tabletop centrifuge (MicroMax Model, IEC, Newark, NY). The supernatant was removed and the cell pellets were resuspended in 150 μ L of a 0.25M Tris-HCl solution (pH 7.8). Cells were lysed by three cycles of freezing in a dry ice/ethanol bath and thawing in a water bath at 37°C water bath (for 3 minutes each cycle). Lysed cell preparations were centrifuged at 15,000 rpm for 5 minutes at 4°C to remove cell debris. Supernatants containing the soluble cell lysate were transferred to a new set of tubes for assaying chloramphenicol acetyltransferase (CAT) activity.

Before performing CAT assays on such cell lysates, the protein content of each lysate was first determined using a commercially-available assay (BioRad Laboratories, Richmond, CA). An amount of each cell lysate containing 100 μ g total protein was then mixed with CAT assay buffer (0.4M Tris-HCl (pH 7.8)/ 0.5mM acetyl-CoA (Boehringer Mannheim)/ 0.1 μ Ci D-threo-(dichloroacetyl-1,2-[14 C]-chloramphenicol) for 15 hours. After this incubation, reactions were stopped by vigorously extracting the reaction mixture with 900 μ L ethyl acetate. The organic and aqueous phases were separated by brief centrifugation at 14,000 rpm for 1 minute, and approximately 800 μ L of the organic phase was transferred to a new set of tubes. Ethyl acetate was evaporated to concentrate the CAT-catalyzed reaction products.

Acetylated and unacetylated chloramphenicol species were resolved by thin layer chromatography using a mixture of 95:5 (v/v) chloroform/methanol as the ascending buffer. Radioactivity from each species so resolved was measured using a Betascope 603 blot analyzer (Betagen, Intelligenetics Inc., Mountain View, CA). The percentage of acetylated counts relative to the total counts was calculated to yield relative CAT activities of each transfectant assayed (all CAT activities expressed herein were calculated on this basis). Each assay was performed in duplicate.

A representation of the results of the above experiment for MG63 transfectant cell lines is shown in Figure 4. The results of a representative experiment are tabulated below:

TABLE I

Promoter	Control	Estradiol		Raloxifene		Tamoxifen	
		Act.	Fold Ind.†	Act.	Fold Ind.	Act.	Fold Ind.
TGF β -1	6.4	4.7	0.7	5.6	0.9	6.9	1.1
TGF β -2	0.8	1.29	1.6	2.3	2.9	2.0	2.6
TGF β -3	0.7	2.1	2.8	5.2	7.3	1.9	2.6

† - Fold induction is calculated based on comparison with control

These experiments demonstrate that transcription of the CAT reporter gene is induced by estrogen and the antiestrogens raloxifene and tamoxifen, with raloxifene displaying a greater potency than estrogen, especially for the TGF β -3 promoter. In contrast, the TGF β -1 promoter region used in this experiment (positions -1032 to +727) showed no response to either estradiol or raloxifene.

TABLE II

For pB-301

	Control	10 ⁻⁹ M	10 ⁻⁸ M	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M
Raloxifene	0.7	2.4	9.6	9.63	9.05	5.25
Estradiol	0.7	1.1	3.2	5.5	5.2	8.0
E2 + Ral	---	17.3	16.0	7.3	9.3	10.5

For pGL2ERELUC:

	Control	10 ⁻⁹ M	10 ⁻⁸ M	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M
Raloxifene	1.7	1.9	1.5	2.0	1.9	1.9
Estradiol	1.7	5.8	5.5	5.9	6.2	6.5
E2 + Ral	---	1.8	1.9	2.1	2.3	2.4

Representative results of these experiments are shown in Figure 5.

These results clearly demonstrate the existence of a distinct promoter region in the TGF β gene promoter that is responsive to antiestrogens. While raloxifene acts as a potent antagonist to transcription initiated by ERE-containing promoters of genes such as the vitellogenin gene, it was found herein to act as a super-agonist in inducing transcription from the TGF β -3 promoter. Interestingly, at low concentrations, raloxifene and estrogen *synergistically* induced transcription from the TGF β -3 promoter in the reporter plasmids of the invention, suggesting that raloxifene-induced gene transcription may be mediated by a novel mechanism.

EXAMPLE VII

Estrogen Receptor Dependent Gene Activation of TGF β -3 by Estrogen and Antiestrogens

It was known in the prior art that the ability of both estrogens and antiestrogens to influence TGF β production is dependent on the expression of estrogen receptor (ER), but the level at which this influence is exerted was not known (i.e. transcriptional, translational or post-translational). A series of experiments were therefore performed to investigate the putative dependence on ER expression of induction of reporter gene expression using the TGF β promoter-containing constructs of the invention. Lack of ER expression virtually abolishes expression of TGF β -3, regardless of the presence of estradiol or raloxifene. Through the use of mutant ER proteins, it has been determined that different domains of the ER molecule are responsible for estrogen and raloxifene induction.

A. ER dependent induction of TGF β -3

Cultures of MG63 were prepared for co-transfection as in Example IV. One such culture was co-transfected with pB2-400 and pRC-VEP, and another was co-transfected with pB2-400 and pRSM (control vector). The ability of the following compounds to induce transcription via the raloxifene responsive element of the TGF β -3 gene was then assayed essentially as described in Example V.

- (a) ethanol
- (b) 17 β -estradiol (10⁻⁷M)
- (c) raloxifene (2 x 10⁻⁶M)
- (d) 17 β -estradiol (10⁻⁷M) and raloxifene (2 x 10⁻⁶M)

The results of one such experiment are set forth in the following Table, and a representative example of a thin-layer chromatogram produced thereby is shown in Figure 6.

EXAMPLE VIII

Activities of Estrogen and Antiestrogens on TGF β -3 Promoter

Transcriptional activation of TGF β promoter-mediated gene expression by estrogen and antiestrogen compounds was found to be concentration-dependent. Cultures of MG63 cells were transiently co-transfected with pB-301 and pRSVER as described in Example V. Such transiently transfected cell cultures were divided into four groups of twelve cultures, and each of the four groups was used to test the ability of one estrogen or antiestrogen compound to induce transcription from the TGF β -3 promoter individually. For each group of twelve cultures, the particular estrogen or antiestrogen compound was tested in replicate cultures at six concentrations, varying in tenfold increments from 10^{-9} M to 10^{-5} M, as well as one set of replicate cultures treated with vehicle only (for a total of twelve cultures per experimental treatment). Hormones were dissolved in ethanol and applied to the cultures in media as described above. The four estrogens and antiestrogens tested were:

- a. 17 β -estradiol (Sigma Chemical Corp., St. Louis, MO);
- b. raloxifene (Eli Lilly, Indianapolis, IN);
- c. tamoxifen (Sigma);
- d. ICI 164,384 (described in European Patent No. EP138504, issued 20 July 1988).

After 24 hours of hormone treatment (or control) the cells were washed, harvested, lysed, and assayed for CAT activity as described in Example V. The results of one such experiment are tabulated below and are depicted in Figure 8:

TABLE V

	vehicle	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
estradiol	0.85	0.96	1.1	1.6	4.3	3.4
raloxifene	0.85	6.9	19.2	18.1	19.6	14.8
tamoxifen	0.85	0.91	1.2	0.98	2.9	13.5
ICI 164,384	0.85	0.89	0.88	10.9	15.2	6.5

Although all estrogens and antiestrogens influence TGF β -3 promoter activity, each compound exhibits its own distinctive dose-response curve. Raloxifene is by far the more potent activator, displaying more than a 20-fold induction of reporter gene transcription and having an ED₅₀ at nanomolar concentrations. In contrast, estradiol showed only a 4-fold induction of reporter gene expression and an ED₅₀ that was two orders of magnitude higher than that of raloxifene. Tamoxifen activates the TGF β -3 promoter only at high levels (i.e., greater than micromolar). ICI 164,384 showed an ED₅₀ of 10^{-7} M, but this compound appears to be much less active at high concentrations (10^{-5} M). These results demonstrate that a novel element has been found in the promoter region of the TGF β -3 gene termed a raloxifene responsive element (RRE). This element induces transcription in the presence of both estrogens and antiestrogens and each of these compounds exhibits a characteristic dose-response profile of transcriptional activation.

EXAMPLE IX

Raloxifene-Mediated Transcriptional Activation of the TGF β -3 Promoter in CHO and MCF-7 Cells

The ability of raloxifene to induce transcription from the TGF β -3 promoter distinct from estrogen-mediated induction was demonstrated in a variety of cell lines.

A. TGF β -3 activation in CHO cells

Cultures of CHO cells were transiently co-transfected with pB-301 and pRSVER as described in Example IV and were used to determine the ability of both raloxifene and estradiol to induce transcription via the raloxifene responsive element. Twelve transiently transfected cultures were treated in replicate with either estradiol or raloxifene at six concentrations varying in tenfold increments from 10^{-9} M to 10^{-5} M (as well as a vehicle only control for a total of twelve cultures). Hormones were dissolved in ethanol and applied to the cultures in media as described above.

3 promoter. These compounds can be distinguished on the basis of a spectrum of *in vivo* activity ranging from uterotrophic (LY112676, LY81099, and LY13314) to anti-uterotrophic (LY113526, LY139482 and LY177366), and included a compound known to be inert *in vivo* (LY98005).

The IUPAC names for the compounds and the U.S. Patents in which they have been claimed are as follows:

5

10

15

20

25

113526	2-(p-hydroxyphenyl)benzo[B]thien-3-yl p-[2-(1-pyrrolidinyl)ethoxy]phenyl ketone	4,133,814
139482	[4-[2-(hexahydro-1H-azepin-1-yl)ethoxy]phenyl][6-hydroxy-2-(4-hydroxyphenyl)benzo[B]thien-3-yl]methanone	4,380,635
177366	[6-(2,2-dimethyl-1-oxopropoxy)-2-[4-(2,2-dimethyl-1-oxopropoxy)phenyl]benzo[B]thien-3-yl][4-[2-(1-piperidinyl)ethoxy]phenyl]methanone hydrochloride	07/902,933, filed July 28, 1992, incorporated by reference)

30

35

40

45

98005	p-hydroxyphenyl 3-(-hydroxyphenyl)benzo[B]thien-2-yl ketone	
112676	(p-hydroxyphenyl) 5-hydroxy-3-phenylbenzo[B]thien-2-yl ketone	4,075,227
81099	p-hydroxyphenyl 3-phenylbenzo[B]thien-2-yl ketone	4,075,227
133314	[3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]phenyl]methanone, methanesulfonic acid salt	

These compounds are depicted in Figure 11.

50

Raloxifene, LY81099, LY98005, LY112676, LY113526, LY13314, LY139482, and LY177366 (Eli Lilly and Company, Indianapolis, Indiana) were assayed to compare their ability to induce transcription from the promoter of the TGF β -3 gene at varying concentrations. Cultures of MG63 cells transiently co-transfected with pB-301 and pRSVER were treated as in Example VI with the above compounds at concentrations of 0M, 10⁻⁹M, 10⁻⁸M, 10⁻⁷M, 10⁻⁶M and 10⁻⁵M. The experimental results are shown in tabular form and depicted in Figure 12.

55

TABLE IX

Vector Plasmid	TGF β -3 Promoter region	Fold induction by raloxifene
pB-499	-499 - +110	6.8
pB-301	-301 - +110	13.1
pB-221	-221 - +110	8.7
pB-91	-91 - +110	10.1
pB-60	-60 - +110	12.5
pB-47	-47 - +110	11.5
TGF β -38LUC	-38 - +110	7.1
TGF β +75LUC	-38 - +75	5.8
TGF β +35LUC	-38 - +35	1.2
pLUC vector alone		0.5

These results localize at least one portion of the raloxifene responsive element to positions +35 to +75 in the TGF β -3 promoter sequence.

B. Nucleotide sequence of the raloxifene responsive element

The nucleotide sequence of the TGF β -3 promoter from position -38 to +110 was depicted in Figure 14. The raloxifene responsive sequence was found above to be the sequence depicted in the Figure in outline form. The open arrow indicates the major transcription start site (+1). The two black arrows indicate the two minor transcription start sites. The "TATA" sequence is shown in the open box. A putative CCCTC-motif is indicated by a series of horizontal arrow heads under the sequence of the putative raloxifene responsive element. See Lobanenkov *et al. Oncogene*, 5, 1743-1753 (1990).

Two conclusions can be drawn from the TGF β -3 analysis. The first is that no palindromic sequences homologous to the ERE was found in this region of the TGF β -3 promoter. This finding is consistent with the results shown in Example VII which demonstrated that DNA binding activity of ER is not required. Second, ER-mediated raloxifene activation of TGF β -3 most likely requires other factors that are capable of binding to the raloxifene responsive sequence. A good candidate for such a protein is the CTCF factor identified by Lobanenkov *et al.*, which is involved in *c-myc* gene regulation. These findings may lead to the identification of other genes as potential raloxifene inducible genes that have raloxifene responsive elements in their promoters. Furthermore, such genes could be used to identify genetic elements having the activity of raloxifene responsive elements for use in the screening procedure set forth in Example XIII.

The raloxifene responsive element of the present invention was used to search the GenBank sequence library; significant homology was found between this element and elements in the following genes:

TABLE XI

	0M	10 ⁻⁹ M	10 ⁻⁸ M	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M
estradiol	1597	1645	1792	1574	1578	2445
raloxifene	1597	1652	1234	1025	1291	5561

These results show that both raloxifene and estrogen have the ability to induce LDL receptor expression. This result provides an explanation of serum lipid lowering effect by estrogen and raloxifene *in vivo* in both animal models and humans.

EXAMPLE XIII

Method for Screening Potential Anti-Osteoporosis Agents

Based on the foregoing Examples, an assay using luciferase as a reporter gene was designed to screen for potential anti-osteoporosis agents.

Cultures of MCF-7 cells were stably transfected with:

1. pTGF β -301LUC;
2. pGL2LUC; or
3. pGL2ERELUC;

using the methods described in Example IV. The cells are then used in the inventive method depicted in Figure 17.

STEP 1. The first procedure that is used in the screening assay is to determine the ability of a compound to induce transcription from the TGF β -3 promoter. The assay is performed essentially as described in Example IX, using MCF-7 cells stably transfected with pTGF β -301LUC. Cell culture and assay conditions are adapted to the 96-well microtiter plate format. Cells are seeded in a 96-well plate at a density resulting in approximately 50% confluency. Test compounds may be selected from a variety of sources, including pharmaceutical research records, chemical manufacturers products lists, and naturally-occurring sources such as fermentation extracts. Cells are incubated in growth media (as described in Example IV) containing the test compound for about 24 hours. Cells are then lysed *in situ* on the plate, and the lysates subjected to both a quantitative protein assay and to the luciferase activity assay. Compounds that induce a greater than two-fold increase in luciferase activity are considered competent for further testing.

STEP 2. Assays are performed with compounds identified as described in Step 1 on cell cultures that have been stably transfected with pGL2LUC to determine whether such compounds are general transcription inducers. As such general transcriptional inducers lack the transcriptional induction specificity required for potential anti-osteoporetics that are modulators of raloxifene-responsive element-dependent gene expression, such general inducers are excluded from further testing.

STEP 3. Compounds having the required transcriptional induction specificity (that is, are capable of inducing transcription induction in pTGF β -301LUC cells without inducing transcription in cells transfected with pGL2LUC) for potential anti-osteoporetics, that are modulators of raloxifene-responsive element-dependent gene expression, are then assayed to determine whether such compounds induce transcription from an estrogen responsive element. Cells stably transfected with pGL2ERELUC are assayed as described in Example VI both in the presence and in the absence of estradiol. Compounds that activate pGL2ERELUC in the *absence* of estrogen are disqualified for further testing, because the capacity of these compounds to induce transcription from an estrogen-responsive element in the absence of estrogen evidences potential estrogenic activity *in vivo*.

STEP 4. Compounds that have fulfilled the criteria of Steps 1 through 3 are then further tested to determine whether such compounds are either anti-estrogenic or non-estrogenic/non-antiestrogenic. To this end, the compounds are assayed in the presence of estradiol in cells stably transfected with pGL2ERELUC. Inhibition of estrogen-induced luciferase activity in this assay indicates that such compounds have anti-estrogenic activity. Both anti-estrogenic and non-estrogenic compounds will be characterized for their dose-response profiles and ED₅₀ values. Further experiments may be done to establish the dose-response profiles of such compounds and to compare them with known anti-estrogens like raloxifene. See Example X.

Following this screening protocol, conventional assays, particularly an *in vivo* assay involving appropriate animal model systems, may be used to further characterize the anti-estrogenic properties of the compounds identified as described herein. Development of such anti-estrogenic compounds having desirable anti-

TABLE XII

compound	TGF β assay ^a	bone density ^b
raloxifene	+	+
088074	-	-
156678	+	+
171147	+	+
309503	-	-

^a "+" indicates the test compound produced a two-fold induction in normalized luciferase activity with an ED₅₀ < 10nM

^b "+" indicates the bone mineral density is statistically higher than ovariectomized animals

The above compounds were also tested for their ability to preserve bone mineral density in ovariectomized rats. Seventy-five day old female Sprague Dawley rats (weight range of 225 to 275 g) were obtained from Charles River Laboratories (Portage, MI). They were housed in groups of three and had *ad libitum* access to food (calcium content approximately 1%) and water. Room temperature was maintained at 22.2°C \pm 1.7°C with a minimum relative humidity of 40%. The photoperiod in the room was 12 hours light and 12 hours dark.

One week after arrival, the rats underwent bilateral ovariectomy under anesthesia [44 mg/kg Ketamine and 5 mg/kg Xylazine (Butler, Indianapolis, IN) administered intramuscularly]. Treatment with vehicle, or a test compound was initiated on the day of surgery following recovery from anesthesia. Oral dosage was by gavage in 0.5 mL of 1% carboxymethylcellulose (CMC). Body weight was determined at the time of surgery and weekly thereafter and the dosage was adjusted with changes in body weight. Vehicle or treated ovariectomized (ovex) rats and non-ovariectomized (intact) rats were evaluated in parallel with each experimental group to serve as negative and positive controls.

The rats were treated daily for 35 days (6 rats per treatment group) and sacrificed by decapitation on the 36th day. The 35 day time period was sufficient to allow maximal reduction in bone density, measured as described herein. The right femurs were excised and scanned at the distal metaphysis 1 mm from the patellar groove with single photon absorptiometry. Results of the densitometer measurements represent a calculation of bone density as a function of the bone mineral content and bone width. Generally, ovariectomy of the rats caused a reduction in femur density of about 25% as compared to intact vehicle treated controls. The results of these experiments are shown in Table XII.

The results of these experiments show that the compounds that potently induce transcription from the raloxifene responsive element of promoter region of the TGF β -3 gene, such as raloxifene, 156678, and 171147, show a preservation of bone density in ovariectomized rats.

The compounds that fail to induce transcription for the raloxifene responsive element, such as 088074 and 309503, also fail to show a statistically significant protection against bone loss in mineral density over ovariectomized rats.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit or scope of the invention as set forth in the appended claims.

(ix) FEATURE:

(A) NAME/KEY: misc_RNA

(B) LOCATION: 1..2

(D) OTHER INFORMATION: /note= "Number 1 corresponds to
-1362 of TGF β -1 promoter"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA

(B) LOCATION: 1363..1365

(D) OTHER INFORMATION: /note= "Corresponds to +1 codon of
TGF β -1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15 GGATCCTTAG CAGGGGAGTA ACATGGATT T GGAAAGATCA CTTGGCTGC TGTGTGGGGA 60
 TAGATAAGAC GGTGGGAGCC TAGAAAGGAG GCTGGGTTGG AACTCTGGG ACAGAAACCC 120
 20 AGAGAGGAAA AGACTGGGCC TGGGGTCTCC AGTGAGTATC AGGGACTGGG GAATCAGCAG 180
 GAGTCTGGTC CCCACCCATC CCTCCTTTCC CCTCTCTCTC CTTTCCTGCA GGCTGGCCCC 240
 GGCTCCATTT CCAGGTGTGG TCCCAGGACA GCTTTGGCCG CTGCCAGCTT GCAGGCTATG 300
 25 GATTTTGCCA TGTGCCCAGT AGCCCGGGCA CCCACCAGCT GGCCTGCCCC ACGTGGCGGC 360
 CCCTGGGCAG TTGGCGAGAA CAGTTGGCAC GGGCTTTCGT GGGTGGTGGG CCGCAGCTGC 420
 TGCATGGGGA CACCATCTAC AGTGGGGCCG ACCGCTATCG CCTGCACACA GCTGCTGGTG 480
 30 GCACCGTGCA CCTGCAGATC GGCCTGCTGC TCCGCAACTT CGACCGCTAC GGCGTGGAGT 540
 GCTGAGGGAC TCTGCCTCCA ACGTCACCAC CATCCACACC CCGGACACCC AGTGATGGGG 600
 35 GAGGATGGCA CAGTGGTCAA GAGCACAGAC TCTAGAGACT GTCAGAGCTG ACCCCAGCTA 660
 AGGCATGGCA CCGCTTCTGT CCTTTCTAGG ACCTCGGGGT CCCTCTGGGC CCAGTTTCCC 720
 TATCTGTAAA TTGGGGACAG TAAATGTATG GGGTCGCAGG GTGTTGAGTG ACAGGAGGCT 780
 40 GCTTAGCCAC ATGGGAGGTG CTCAGTAAAG GAGAGCAATT CTTACAGGTG TCTGCCTCCT 840
 GACCCTTCCA TCCCTCAGGT GTCCTGTTGC CCCCTCCTCC CACTGACACC CTCGGAGGC 900
 CCCCATGTTG ACAGACCCTC CTTCTCTAC CTTGTTTCCC AGCCTGACTC TCCTTCCGTT 960
 45 TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG 1020
 TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG TCTGCTGCTG 1080
 50 CCGGCTTTGG TGGCGCTTGG CCGCCAGGAG GCAGCACCCCT GTTTCGGGGG CGGAGCCGGG 1140
 GAGCCCGCCC CCTTTCCCCC AGGGCTGAAG GGACCCCCCT CGGAGCCCGC CCACGCGAGA 1200
 TAGAGAGGCT GGGCGAGCCC CCGCATGCCC TCCCCTAGG GGGCGCCCCC GGTCCCCCCC 1260

(ix) FEATURE:

(A) NAME/KEY: misc_RNA

(B) LOCATION: 3635..3980

5 (D) OTHER INFORMATION: /note= "CDS, Codon start = 1"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA

(B) LOCATION: 1..2

10 (D) OTHER INFORMATION: /note= "Number 1 corresponds to
TGFB-2 -2277"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15	AAGCTTTTAC CAATACCTCC CGTCTTACCC CTCCTGGGCT TTGGGGAAAT TAAAGTAGCC	60
	TCTTATGAGT AAGTCAGGGG TCTCAGGTCT AAGGAGTTGT TAAGTGAGCA ATAGGTACTC	120
	AGTAACTAA GTATTATGAA CAAAAGTGTG TATGTCTATG TCAGGAAGAG GGGTGGCCAT	180
20	CAGAATTTAT GGCTTGGGCT GTTTCCTAGA AGTGATGTAA TGAACTTTGT CTACTCTATC	240
	CACACTCTAA TCTGAATCTA CTTAAGGTGC ATCAGTGTCT GTACCAAGAA GGTGTGTCTAT	300
	AAACATGAAA GATGGATGCT CACTGGCTTG TGGAAGCTGA ACCTGTATCC TCAGAAAATA	360
25	CAGTGATAGC TAATTCAGGT AACCAGCCAT ATTCCACAGC AGCATCTTCT CTCAGTAGCT	420
	CTGGTTTGA GCTCCTGCTC TGTGTCTATA ATGGCCACAG GTGTAAGAAT ATTCACCTTTT	480
30	TGTCCAATCT GTAGAGCTAG CCTACTGCAG TTCTCAAAC TGAATCAGAG GGAGGACCTA	540
	ACTGGATGAA ACTACTAGTC TGACAGTAGC GCCTCTTGAT TATCTTTTTT TTGGGCTACT	600
	GGGATGGTAG CTTTGCTTCA ACTCAAAACT GGTATCAAGG AAAGGAACCT GCTGGTGCTG	660
35	ATTTATACAT AATTTTTAGA ATTATTCAGA AGTGGGTTGG AACAATTATT TTATTCCAGA	720
	GTTTTTCAAT GTGTGATAAT GGAAAAAATT CTGTATTCAA GGGAGTTTGG AAAATGCTGG	780
	GTTAAAAGAG TGAAAAGGTT TTCTCTTCTA CAGGAGTTTC AGAGCCTTTA ACATGATAAT	840
40	GTTCCAGAAT GAGGAATCTA AGAGGACAGG AGAGTACCCA GTATCTCCCA AACTTGTTTG	900
	ACTCCAGAAT TCCTGTTTGT CAGAACATAT TCTGGGACCA TTGTTTCTCA GAAGTACATA	960
45	GTAGGTAAGA ACATAGTGGG TCTGACTGC AAAAATCCAG CTCTAGCACT TACTGTGGTC	1020
	TGGAACAAAG TACTTAACTT CTTGTACCT CAGTCTCT ATCTTCCAGA TATGATAAT	1080
	AAGACCCACT TTATAGGTTT ATAGTGAAGA TTAAATGACC ATACACAACA CACATCAAAT	1140
50	TACTAAGTGT AGTATATGTT AGCTATTATT ATTTTATTTA TTCAGTGCTC TACTAATAAC	1200
	CTAGTCTCA TACATCACTT AAGTATAATT TCAAAAGTCA TAGAAAGTTC TTCTTACCTT	1260
55	TTCTGAACTC AGGAACATCT GAAGTAGAGA ACAGTATAGA GATCTTGGGT TTGGGAGTAC	1320

	CACACACACA CACACACACG CACGCACACA CGTGTCGTTC TCTGCTCCGG AGCTGCTGCT	3060
5	GCTCCTGCTC TCAGCGCCGC AGTGGAAGGC AGGACCGAAC CGCTCCTTCT TTAAATATAT	3120
	AAATTTTCAGC CCAGGTCAGC CTCGGCGGCC CCCCTCACCG CGCTCCCGCC CCTCCCGTCA	3180
	GTTCCGCCAGC TGCCAGCCCC GGGACCTTTT CATCTCTTCC CTTTGGCCG GAGGAGCCGA	3240
10	GTTTCAGATCC GCCACTCCGC ACCCGAGACT GACACACTGA ACTCCACTTC CTCCTCTTAA	3300
	ATTTATTTCT ACTTAATAGC CACTCGTCTC TTTTTTCCC CATCTCATTG CTCCAAGAAT	3360
	TTTTTCTTTC TTA CTGCGCA AAGTCAGGGT TCCCTCTGCC CGTCCCTAT TAATATTTCC	3420
15	ACTTTTGGAA CTACTGGCCT TTCTTTTTA AAGGAATCA AGCAGGATAC GTTTTCTGT	3480
	TGGGCATTGA CTAGATTGTT TGCAAAAGTT TCGCATCAAA AACAACAACA ACAAAAAACC	3540
20	AAACAACCTCT CCTTGATCTA TACTTTGAGA ATTGTTGATT TCTTTTTTTT ATTCTGACTT	3600
	TTAAAAACAA CTTTTTTTTT CACTTTTTTA AAAAATGCAC TACTGTGTGC TGAGCGCTTT	3660
	TCTGATCCTG CATCTGGTCA CGGTGCGGCT CAGCCTGTCT ACCTGCAGCA CACTCGATAT	3720
25	GGACCAGCTC ATGCGCAAGA GGATCGAGGC GATCCGCGGG CAGATCCTGA GCAAGCTGAA	3780
	GCTCACCAGT CCCCCAGAAG ACTATCCTGA GCCCGAGGAA GTCCCCCGG AGGTGATTTT	3840
30	CATCTACAAC AGCACCAGGG ACTTGCTCCA GGAGAAGGCG AGCCGGAGGG CGGCCGCCTG	3900
	CGAGCGCGAG ACGAGCGACG AAGAGTACTA CGCCAAGGAG GTTTACAAAA TAGACATGCC	3960
	GCCCTTCTTC CCCTCCGAAA GTAAGTACTT ATTTGACTT CCATCCCCTG AGGTTTAGCT	4020
35	CTGCCCCGAG CTCTCAAAAC CGCAGCAGCT CCCGGGATCG CCCTTCCCTC TGCCGGTTCC	4080
	CGTTCGCTCT TTTCCCGTTC TCCTGTCTT CACCCACCA CCTCCTTTT AGTTGTAGTC	4140
	TTGAGGCCAT CAGGCTTTAA AATGTTTACT TTCTACTTTA TTTCTCCAT CTTTCCCTG	4200
40	CCCCCTAACA ATGCGGTTCT TTAAAAGGCG TTATTCTCTT TTTCTCTTCC CTGAAGTTCT	4260
	TTAGTCGGCC ACCAGCTAAG GAGTCAGCCC CACTCTGTCA AACTAGAGGT GCTCCCAGGG	4320
45	GCAGAGTTAA ACTGAGGAAT CTTCGTAGGT TTGTTTTCTT TGCTCCGATT GGCGTGGAGC	4380
	CTCTCTTCTT TTTCTCTTCC TTTCTCTTCC TTTCTCTTCC TTTCTCTTCC TTTCTCTTCC	4440
	TTCTCTTCTT TTTCTCTTCC TTTCTCTTCC TTTCTCTTCC TTTCTCTTCC TTTCTCTTCC	4500
50	ACCCTAAAAA TGTACGATC GCCGAGACAA AACCCGGTTT GGAGACCCCT CGAGAAACCT	4560
	ACCTGGCCCT CAGTCCTTGA TGTATACTT GCTATACCTA GGTGTTTCAT TACCCAGCGG	4620
	CAAAATCTTA TAA CAGCTT CTTTCTTAC TTAACCTTGA GCGGAGAAAG GACAA TCGG	4680
55		

(ix) FEATURE:

(A) NAME/KEY: misc_RNA

(B) LOCATION: 3301..3303

(D) OTHER INFORMATION: /note= "CDS Start, codon start = 1,
translation M"

(ix) FEATURE:

(A) NAME/KEY: TATA_signal

(B) LOCATION: 2170..2176

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1896..2306

(D) OTHER INFORMATION: /note= "pB-301 -301 to +110"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1976..2306

(D) OTHER INFORMATION: /note= "pB-221 -221 to +110"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 2106..2306

(D) OTHER INFORMATION: /note= "pB-91 -91 to +110"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 2137..2306

(D) OTHER INFORMATION: /note= "pB-60 -60 to +110"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 2150..2306

(D) OTHER INFORMATION: /note= "pB-47 -47 to +110"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 2159..2306

(D) OTHER INFORMATION: /note= "pB-38 -38 to +110"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 2159..2271

(D) OTHER INFORMATION: /note= "TGFB-3 position -38 to
+75"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 2159..2231

(D) OTHER INFORMATION: /note= "TGFB-3 position -38 to
+35"

	ACCATTTTTTC	ACTGCCCCCTG	GAGCGTCTCC	AGGCTTCTGC	CCGCCTCCCG	ACTCCGATCT	1680
5	TGTCAATGAA	GAATCGGGCC	AGGATCGCCG	CGGAGCGGAC	GCCGACCCTC	CGACCCGGCT	1740
	CGCAGGCTGG	GAGTCCCCCTC	TGCGAGGCTG	GCATGGCCGC	CCCTACCGGG	TCCCGCGCCC	1800
	TCTGCGGACC	CTCCCCGGGT	TGGGCCTGGC	CGCGGGCGGC	CCCGGGACCG	GGGGACCAGG	1860
10	AGGGAGAGTA	GACCGGGCCG	GACGGCGCGG	ACTGACAGCT	GGCGAGAGGG	CGCCGGGGCT	1920
	GGGGGAAAGG	GAGGGAGGGG	GCTCATCGGA	GTAACTTTCC	AGAAAAACAC	CAACGTGTGG	1980
	CAGGAGTGAT	TCCAAGAGGG	GAAAAAAGT	TCAGCTACCA	CGTCGAACGA	GAGGACTCGC	2040
15	AAAGTATTTT	TCAAAAGGGC	TGGGCTTTTC	CTGTGCCTGT	TTAAAACATT	AACATCGTGC	2100
	AGCAAAAGAG	GCTGCGTGCG	CTGGTCCCTC	CCTCCCCCAC	CCCAGGCCAG	AGACGTCATG	2160
20	GGAGGGAGGT	ATAAAATTTT	AGCAGAGAGA	AATAGAGAAA	GCAGTGTGTG	TGCATGTGTG	2220
	TGTGTGTGAG	AGAGAGAGGG	AGAGGAGCGA	GAGGGAGAGG	GAGAGGGAGA	GAGAGAAAGG	2280
	GAGGGAAGCA	GAGACTCAAG	TCCAAGGGAA	TGACCGAGAG	AGGCAGAGAC	AGGGGAAGAG	2340
25	GCGTGCGAGA	GAAGGAATAA	CAGCAGCTTT	CCGGAGCAGG	CGTGCCGTGA	ACTGGCTTCT	2400
	ATTTTATTTT	ATTTTMTTCT	CCTTTTTATT	TTTTAAAGAG	AAGCAGGGGA	CAGAAGCAAT	2460
	GGCCGAGGCA	GAAGACAAGC	CGAGGTGCTG	GTGACCCTGG	GCGTCTGAGT	GGATGATTGG	2520
30	GGCTGCTGCG	CTCAGAGGCC	TGCCTCCCTG	CCTTCCAATG	CATATAACCC	CACACCCACG	2580
	CCAATGAAGA	CGAGAGGCAG	CTGAAAAAGT	CATTTAGAAA	GCCCCCGAGG	AAGTGTA AAC	2640
35	AAAAGAGAAA	GCATGAATGG	AGTGCCTGAG	AGACAAGTGT	GTCTGTACT	GCCCCACCTT	2700
	TAGCTGGGCC	AGCAACTGCC	CGGCCCCGCT	CTCCCCACCT	ACTCACTGGT	GATCTTTTTT	2760
	TTTTTACTTT	TTTTTCCCTT	TTCTTTTCCA	TTCTCTTTTC	TTATTTTCTT	TCAAGGCAAG	2820
40	GCAAGGATTT	TGATTTTGGG	ACCCAGCCAT	GGTCCTTCTG	CTTCTTCTTT	AAAATACCCA	2880
	CTTTCTCCCC	ATCGCCAAGC	GGCGTTTGGC	AATATCAGAT	ATCCACTCTA	TTTATTTTPTA	2940
	CCTAAGGAAA	AACTCCAGCT	CCCTTCCCAC	TCCCAGCTGC	CTTGCCACCC	CTCCCAGCCC	3000
45	TCTGCTTGCC	CTCCACCTGG	CCTGCTGGGA	GTCAGAGCCC	AGCAAAAGCT	CTTTTCTTCT	3060
	CTTTTCTTCT	CTTTTCTTCT	CTTTTCTTCT	CTTTTCTTCT	CTTTTCTTCT	CTTTTCTTCT	3120
50	AGGCTTCTCT	GGAAGTCTCT	AAGCTCTCTG	ACTGCAGTGA	GTTCATGCAC	CTTCTTGCCA	3180

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCTCTCCA GTCGCGA

17

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCGAGAAAAG TCAGGTCACA GTGACCTGAT CAAAC

35

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

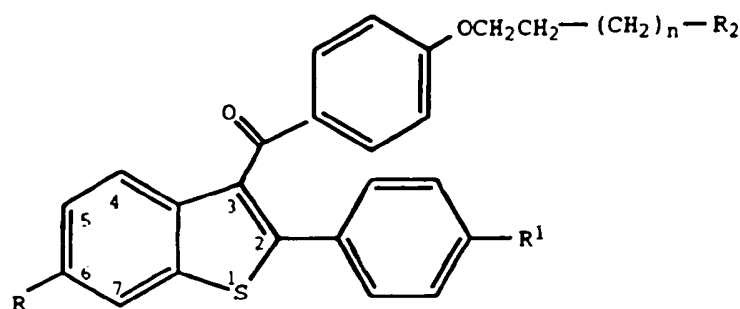
TCGAGTTTGA TCAGGTCACCT GTGACCTGAC TTTTC

35

Claims

A nucleic acid comprising a raloxifene responsive element isolated from a promoter region of a TGF β gene wherein the human TGF β gene is TGF β -2 or TGF β -3.

2. A nucleic acid according to Claim 1 wherein the nucleotide sequence of the nucleic acid comprises a sequence selected from the group consisting of sequences corresponding to 1896 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 1976 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2106 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2137 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2150 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2159 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2159 to 2271 shown in Figure 3 and SEQ ID NO:3, and sequences



(I)

wherein

n is 0, 1 or 2;

R and R^1 , independently, are hydrogen, hydroxyl, C_1 - C_6 -alkoxy, C_1 - C_6 -acyloxy, C_1 - C_6 -alkoxy- C_2 - C_6 -acyloxy, R^3 -substituted aryloxy, R^3 -substituted aroyloxy, R^4 -substituted carbonyloxy, chloro, or bromo;

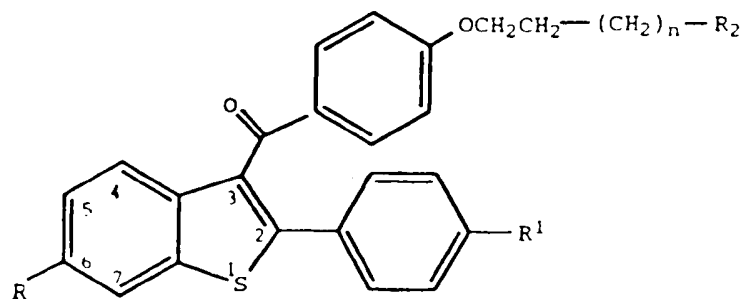
R^2 is a heterocyclic ring selected from the group consisting of pyrrolidino, piperidino, or hexamethyleneimino;

R^3 is C_1 - C_3 -alkyl, C_1 - C_3 -alkoxy, hydrogen, or halo; and

R^4 is C_1 - C_6 -alkoxy or aryloxy; or

a pharmaceutically acceptable salt thereof.

8. A method for treating bone fractures which comprises administering a compound that, when bound to an estrogen receptor, potentially induces transcription from a raloxifene responsive element of a promoter region of a $TGF\beta$ -3 gene, provided the compound is other than a compound of the formula



(I)

wherein

n is 0, 1 or 2;

R and R^1 , independently, are hydrogen, hydroxyl, C_1 - C_6 -alkoxy, C_1 - C_6 -acyloxy, C_1 - C_6 -alkoxy- C_2 - C_6 -acyloxy, R^3 -substituted aryloxy, R^3 -substituted aroyloxy, R^4 -substituted carbonyloxy, chloro, or bromo;

R^2 is a heterocyclic ring selected from the group consisting of pyrrolidino, piperidino, or hexamethyleneimino;

R^3 is C_1 - C_3 -alkyl, C_1 - C_3 -alkoxy, hydrogen, or halo; and

R^4 is C_1 - C_6 -alkoxy or aryloxy; or

a pharmaceutically acceptable salt thereof.

9. A method for inducing bone formation in a mammal which comprises administering a compound that:
- potentially induces transcription from a raloxifene responsive element of a promoter region of a $TGF\beta$ -3 gene;
 - does not induce transcription from a mammalian promoter not having a raloxifene responsive element;

FIG. 1A

-1362 GGATCCTTAGCAGG 12 TAACATGGATTGGAAAGATCACTTTGGCCTGCTGTGTGGGATAGATAAGACGGTGGGAGCCTAGAAAGAGGCTGGGCTTG
-1262 AAACCTCTGGGACAG 121 CAGAGAGGAAAGACTGGGCCCTGGGTCTCCACTGATATCAGGGAGTGGGGAATCAGCAGGAGTCTGGTCCACCCCATC
-1162 CCGCCTTTCCCGTCGCTCTCTCCAGGCTGGCCCCGGCTCCATTTCACGGTGTGCTCCAGGACAGCTTTGGCCCCGCTGCCAGCTTCCAGGCTATG
-1062 GATTTGCCCATGTG 121 TAGCCCCGGCACCCACCAGCTGGCCTGCCCCACCGTGGCGGGCCCCCTGGGCAAGTTGGCGAGAACAGTTGGCACGGGCTTTCTGT
-962 GCGTGGTGGGGCCG 121 GCTGCATGGGGACACCATCTACAGTGGGGCCGACCGCTATCGCCTGCACACAGCTGCTGTGTGGCACCGTGCACCTGGAGATC
-862 GGCCTGCTGCTCAG 121 TTCCACCGCTACGGCGGTGGAGTGCTGAGGGACTCTGCTCCTCCAAGTCAACCATCCACACCCCGACACCCAGTGATGGGG
-762 GAGGATGGCACAGT 121 AAGAGCACAGACTCTAGAGACTGTACAGAGCTACCCCAAGCTAAGGCATGGCACCCGCTTCTGTCTTTCTAGGACCTCGGGGT
-662 CCGCTGTGGCCCCAG 121 CATACTCTGTAAATTGGGGACAGTAAATGTATATGGGTGCGAGGGTGTTCAGTGACAGGAGGCTGCTTAGCCACATGGGAGGCTG
-562 CTCAGTAAAGGAGAG 121 TTCTTAGAGGTGTCTGCTCCTGACCCCTTCCATCCCTCAGGTGTCTCTGTGGCCCCCTCCCTCCCACTGACACCCCTCCGGAGGC

FIG. 1C

639 GGTTATTTCCTGGGATGAGACACCCCGGGTCCAGCCCTCCCTCCACCACTGGGCCCCCTTCTCCCTGAGGAGCCTCAGCTTTCCTCGAGGCCCTCC

739 TACCTTTTGGCGGAGATCCAGCCCCCTGCAGGGGGGGGGCCCTCCCCCACACACAGCCCTGTTCCGGCTCTCGGCACATGCCGGGGGGGGCGCCCTCCC

839 CCATG
Met

FIG. 2A


2277 AAGCTTTTACCAATACCTCCCGTCTTACCCCTCCTGGGCTTTGGGAAATTAAAGTAGCCCTCTTATGAGTAAGTCAG
 -2200 GGGTCTCAGGTCCTAAG AGTTGTTAAGTGAGCAATAGCTACTCAGTAAACTAAGTATTATGAACAAAAAGTGTGTATGTCTATGTCTCAGGAAGAGGGGTGGC
 -2100 CATCAGAAATTATGCTA  TGGGCTGTTTCCCTAGAAAGTGATGTAATGAACCTTTTGGCTACTCTATCCACACTCTAATCTGAACTCTACTTAAGGTGCATCAGTG
 -2000 TCTGTACCAAGAAATCTCTATAAACATGAAGATGGATGCTCACTGGCTTGTGGAAAGCTGAACCTGTATCCCTCAGAAAAATACAGTGTATAGCTAATTCA
 -1900 GGTAACCAAGCCATATTACAGAGCATCTTCTCTCAGTAGCTCTGGTTTGGAGCTCCCTGCTCTGTGTCTATAATGGCCACACAGGTGTAAAGAAATATTCACT
 -1800 TTTTGTCCAAATCTGTAAGCTAGCCCTACTGCAGTTCTCTCAAACCTGAACCTCAGAGGGAGGACCTAACTGGCATGAACCTACTAGTCTCAGAGTAGCGCCCTCTT
 -1700 GATTATCTTTTCTCTGCTACTGGGATGGTAGCTTTTCTTCAACTCAAAACCTGGTATCAAGGAAGAACCTGCTGCTGATTTATACATAAATTTT
 -1600 AGAATTATTCAGAAATTTATTTTATTTCCAGAGTTTTTTTCAATGTGTGATAATGGAAAAAATTTCTGTATTCAAGGGAGTTTTGGAAAAATGC
 -1500 TCGGTTAAAGAGTATAAAGGTTTTCTCTTCTACAGGAGTTTTTCAGAGCCTTTTAACATGATAATGTGTTCCAGAAATCAGCAATCTTAAGAGGCACAGGAGACTAC
 -1400 CCAGTATCTCCCAATATGCTTTGACTCCAGAAATTCCTGTTTGTCTCAGAACATATTCTCTGGACCATTTGTTTTCTCAGAAAGTACATAGTAGGTAAGAACAATAGT

FIG. 2B

-1300 GCATCCTGACTGCA/AA TCCAGCTCTACCACTTACTCTGGTCTCGAACAAGTACTTAACCTTCTTTGTACCTCAGTCTCCTCATCTGCCAGATATGGAT

-1200 AATAAGACCCACCTTATCGTTCATAGTGAAGATTAAATGACCATACACAACACACATCAAAATTACTAAGTGTAGTATATGTTAGCTATTATTATTTTAT

-1100 TTATTGAGTGGCTCTCTTATAACCTAGGCCCCATACACAACACTCAAGTATAATTCCAAAAGTGATAGAAAGTTCTTTGTGACTTTTCTGAACTCAGGAACA

-1000 TCTCAAGTAGAGAA/AAATAGAGATCTTTGGGAGTACATTCAACAGAGTTTCCAGTTTAAATCATCTGTCTGGTCAGTATGGCTCCAGAGTCA

-900 TGCCGAAATGAAATCTGACTTTTGAGTAACTAAAGGTAAATATAAGAAAAAGGGAAGGTGGAACAGTGGTAAGAGTTATTCTGTATTCTCATCTAATTTTA

-800 AGACTTAGTTGAAATG/AAATGTCAAGTTATGAGTAGTGTAGAACAAAGTAGACATCAAAACACTTAATAATTCACGCTTCCTGGATTATGCTATGGAAGA

-700 ATCAAGTTGGTGGAA/AA GTTAGCCTAGCAAGAAGGTCAAGAAGAGAAAGCCATACAAGAAGTGGCTTAGGCAGCA/AAATTATAAAGGTGACCATTCAT

-600 TCAATCAGTAA/AA/AAAGTATACCTTATTCTTTAGGTAA/AAATTGATGGATCTCTGTTTCCAGCAGTTCACAAACAGAGGGGTACATTGTAAACAA

-500 CAAACTAACAAAAT/AA TCTGGGATGGCAACCTGCTAAGGTATCCACAGAAATAAGAGGTAGGACATGAATTTAAAGATTGGGAAGGTATGTCCTTCAGT

-400 ACTGGCCCTGGCCCTAGAGACTAGTGGCCCTCCATAGGGGTGGGTGTCACACACATAATACAGGAGGGAAGCCTTCCCTTCTAGAGCAAGTCAATTCAGCCTT

FIG. 2C

- 300 GCGAGGGCTGTGACGGA CTACACTAAGTAAAAACGGGAGACTTTCATTGTCTCTTCAACAGACAGCCTGTCTCCAAAATGACTCGGAAAGTAAATACCGTAAATCACT
 - 200 GTTGTCAAGGGGGCGGCA TCCACCTCCTTCTCTCCCTTACCCACAGCGGTCCACATTTCCACACTCCCTACACGGTTCCGGGAGAGCTCGTGTCTCTAAAGTAA
 - 100 CGAGAGGACTTCTTA GTAAATCCTAGCACGTCACCTTTGTTGAAGGCAGACACGTCGTTTCAGAGAGAACTTATAAACTCTCCCCCTCCCCCGGAAGATCGCTG
 - 1 ATGTTATTGGCTGCA CAGAAAGGTCTTGGCGAGCGAGCTCCAGAACGTCCTGACAAAGAGAAAGACAGATTGAGATAGAGATAGAAAGAGAAAGAGAGAA
 101 AGAGACAGCAGAGGCA GAGCGCAAGTGAAAGAGGCGAGGCGAGGGGGATGGAGCATATTACGTGACCGGGCCTAGGGAGTCA TCCAGGAACAAACTGAG
 201 GGGCTGCCCCGGCTGCA CACAGGAGGAGACAGAGAGGATCTATTTTAGGGTGGCAAGTGCCTCCTACCTAAGCGAGCAATTCCACGTTGGGGAGAAAGCCA
 301 GCAGAGGTTGGGAAGG GTGGGAGTCCAAAGGGAGCGCCCCCTGCCCAACTCCCTCTCAGGAATAAAACTCCCCAGCCAGGGTGTCTCGCAAGGGCTGCCGTTGTGA
 401 TCCCGCAGGGGCTGAA CCAACCGCGCAGGGCTGATCGTATGTGGCTGGGTTCGCCGTTTGGAGCAAGAGAAAGGAGGAGCGAGCAAGAGAGGGAGGCTGCGAGGC

FIG. 2D

[illegible]Met His Tyr Cys Val Leu Ser Ala Phe Leu Ile
ATG CAC TAC TGT GTG CTG AGC GCT TTT CTG ATC

FIG. 3A

1 CAGTAGTAGC TTCCAGAACT TGCTTAGCAC CTGAATCACG TGTGAGGTTT
51 GTAAAGAAAC AGAGATGCCA GGGCCTCAGC TCTGGAGACT GATTGGTAGA
101 GGTGGAGTCC AAAAAAGTAT AACTTTAATA ATTTTCCTTC CTATCTTCAA
151 CTGTCTGCTC AAAGGCCTTC CCTTATCACC CTATTTGAAA CTGCAACATC
201 CCCCAACCTA GGCACACCCC ATCCTCCTTC CCTGCTTGAT TTTCTGCCAC
251 ACCACATTTG TTTGTTTGCT TGTCTGTTTG AGACACGGTC TTGCTCTGTC
301 GTCCAGGCTG GAGTGCAGTG GTGCAATCTT GGCCCCCTGT AAACCTCGCCT
351 CCCTGGCTCA AGTGATTATC CTGCTCAGCC TCCCAAGTAG ATGCGTGCGC
401 CAACATGCCG GGCTAATTTT TCCATTTTTT TGTAGAGACT GGGTTTCGCC
451 GTGTTGCTGG GGCTGGTCTC GAATTCCTGA GCTCAAGTAA TCCTCCTGCA
501 TGGGCCTCCC CAAATGCTGG GATTACAGGC GTGAGCCACT GCACCTGGCT
551 CAGCACTTTT TACCGTACTA CATCATTTAC ATATTTATTT AGTTTATCGC
601 CTCCTCCACT GCCCCACCCC TGCCTCTAAA TAAAATTTCC CTGAGGGCAG
651 GAGTTTGTG TGTGAGTCA ATATCTTAT TGGAGCTTAC ATTTGCTG
701 GGTATATAGA AACATTAAAC TTTTCTGAA ATTCAGAGG CAGTATAGCA
751 TAGTAATTAA GTCCAGAATC TGGCAACGTC CTGGGTGCAA ATCCCAACAG
801 CTGACACCTA ATAACATGT GACCTTGGGC AAGTTACTTT TAAAGTTTCT

FIG. 3B

851 ACCCCTAGGT TTCCCATTTGG TTTTGCAAAT GAAAGTAATG CCTACCCAAG
901 CTAGATAGCC TGTGTAAATA TCGCCTCCAT CACTCACAAG CAGTGTGGTC
951 TGTAAAAAAA AAAACAAAAA ACTCTATGCC TCAGTTTCCT CATCCGTAAA
1001 AGTGACCCAC CGCTGTGCTG GGATACAGAG AACAGCCCCT TCAGTTAGTG
1051 GCCTGGAAGC CAGCCTCTCA GAAAGGGTCC AGGAAGGCTG GAGTGAGATG
1101 GGGTGGAGCG GCACTCACTC TCAGGAAAGT TCAGTTCAGA GGCAAGCCCT
1151 GTGTTGCGGG GTGCGGGGAG CCACGTGCCC TACCCTCCCT TGGCTGCTCG
1201 TGGGAAAAGG CCTAGAGGTT CGGGCCGAGA AGAGGAGCGA AAGCACAGAG
1251 CCGACTTCCC CTCACCCATC TGGGAAATGG CTCGGGCCAA CTGCTGACTT
1301 CGCGCTCGCT GGCCGACGTC CTGCGGAGAC CTCGGCGGGG AGGGAGGCTG
1351 AACATCTGGA TGACATTTCT GCGAGAGAGC GGCTCCGGAG CGGCGGTCCG
1401 GGAGGGAGAG CTGCTCGTGC GCACGTCGGG CCGGGAGGGA GGCGATTCTT
1451 CGGGGCCTGG GTCTTGTTTT TCTCGCTCTC TACCGCAGCC CCTTCTCCCG
1501 TCCCTCAGG TACCTGGG AGCCCGCAG TCCGAGCT TCCGCTCC
1551 CGACCAGCCG AGCTCCTTCA CTGGCGGCCT CCGCTCGCCA GAGGGCACCC
1601 TCGATCTTCT GAAAAAGGCG ACCATTTTTC ACTGCCCCTG GAGCTATTC
1651 AGGCTTCTGC CCGCCTCCCC ACTCCGATCT TGTCAATGAA GAATCGGGCC

FIG. 3C

1701 AGGATCGCCG CGGAGCGGAC GCCGACCCTC CGACCCGGCT CGCAGGCTGG
 1751 GAGTCCCCTC TGCAGAGGCTG GCATGGCCGC CCCTACCGGG TCCCGCGCCC
 1801 TCTGCGGACC CTCCCCGGGT TGGGCCTGGC CGCGGGCGGC CCCGGGACCG
 1851 GGGGACCAGG AGGGAGAGTA GACCGGGCCG GACGGCGCGG ACTGACAGCT
 1901 GGCGAGAGGG CGCCGGGGCT GGGGGAAAGG GAGGGAGGGG GCTCATCGGA
 1951 GTAAC TTTCC AGAAAAACAC CAACGTGTGG CAGGAGTGAT TCCAAGAGGG
 2001 GAAAAAAAGT TCAGCTACCA CGTCGAACGA GAGGACTCGC AAAGTATTTT
 2051 TCAAAAGGGC TCGGCTTTTC CTGTGCCTGT TTAAAACATT AACATCGTGC
 2101 AGCAAAAGAG GCTGCGTGCG CTGGTCCCTC CCTCCCCCAC CCCAGGCCAG
 2151 AGACGTCATG GGAGGGAGGT ATAAAATTTT AGCAGAGAGA AATAGAGAAA
 2201 GCAGTGTGTG TGCATGTGTG TGTGTGTGAG AGAGAGAGGG AGAGGAGCGA
 2251 GAGGGAGAGG GAGAGGGAGA GAGAGAAAGG GAGGGAAGCA GAGAGTCAAG
 2301 TCCAAGGGAA TGACCGAGAG AGGCAGAGAC AGGGAAGAG GCGTGCGAGA
 2401 ATTTTATTTT ATTTTCTTCT CCTTTTATT TTTTAAAGAG AAGCAGGGGA
 2451 CAGAAGCAAT GGCCGAGGCA GAAGACAAGC CGAGGTGCTG GTGACCTTGG
 2501 GCGTCTGAGT GGATGATTGG GGCTGCTGCG CTCAGAGGCC TGCTTCCCTG

-91
 -38
 -301
 -221
 -60
 -47
 +1
 +35
 +75
 +110

FIG. 3D

2551 CCTTCCAATG CATATAACCC CACACCCCAG CCAATGAAGA CGAGAGGCAG
2601 CTGAAAAAGT CATTTAGAAA GCCCCCGAGG AAGTGTAAC AAAAGAGAAA
2651 GCATGAATGG AGTGCCTGAG AGACAAGTGT GTCCTGTACT GCCCCACCTT
2701 TAGCTGGGCC AGCAACTGCC CGGCCCCGCTT CTCCCCACCT ACTCACTGGT
2771 GATCTTTTTT TTTTACTTT TTTTCCCTT TTCTTTTCCA TTCTCTTTTC
2801 TTATTTTCTT TCAAGGCAAG GCAAGGATTT TGATTTTGGG ACCCAGCCAT
2851 GGTCTTCTG CTTCTTCTTT AAAATACCCA CTTTCTCCCC ATCGCCAAGC
2901 GGCGTTTGGC AATATCAGAT ATCCACTCTA TTTATTTTTA CCTAAGGAAA
2951 AACTCCAGCT CCCTTCCCAC TCCCAGCTGC CTTGCCACCC CTCCCAGCCC
3001 TCTGCTTGCC CTCCACCTGG CCTGCTGGGA GTCAGAGCCC AGCAAAACCT
3051 GTTTAGACAC ATGGACAAGA ATCCCAGCGC TACAAGGCAC ACAGTCCGCT
3101 TCTTCGTCCT CAGGGTTGCC AGCGCTTCCT GGAAGTCCTG AAGCTCTCGC
3151 AGTGCAGTGA GTTCATGCAC CTTCTTGCCA AGCCTCAGTC TTTGGGATCT
3201 GGGGAGGCCG CCTGGTTTTT CTCCCTCCTT CTGCAAGTCT GCTGGGGTCT
3251 CTTCTCTCC AGGCCCTTGCC GTCCCCCTGG CCTCTCTTCC CAGCTCACAC
3301 ATG

FIG. 4

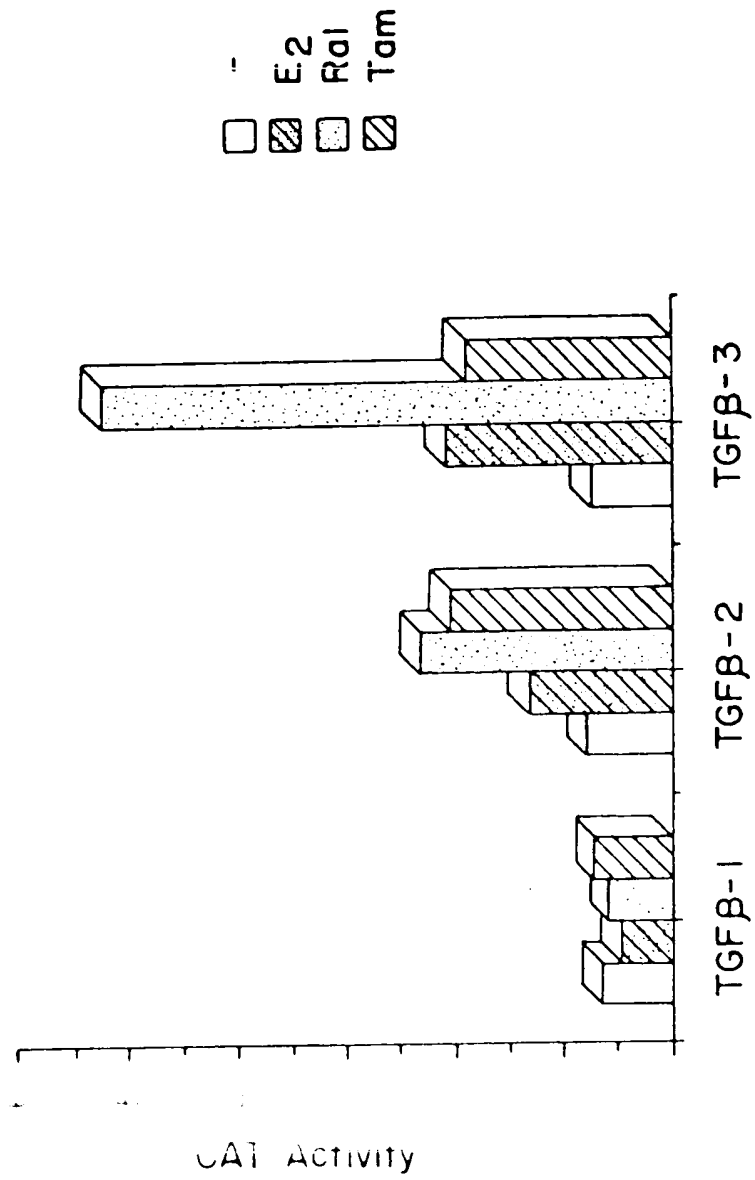


FIG. 5A

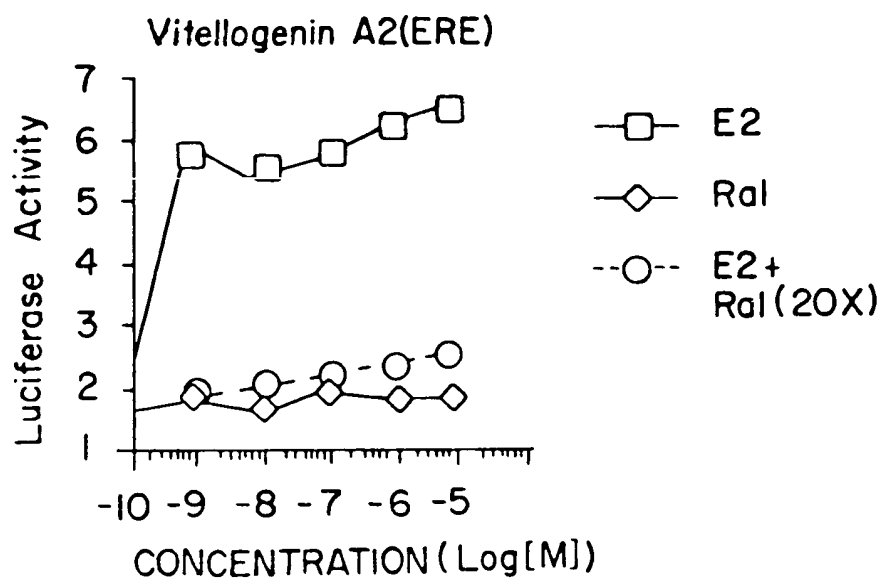


FIG. 5B

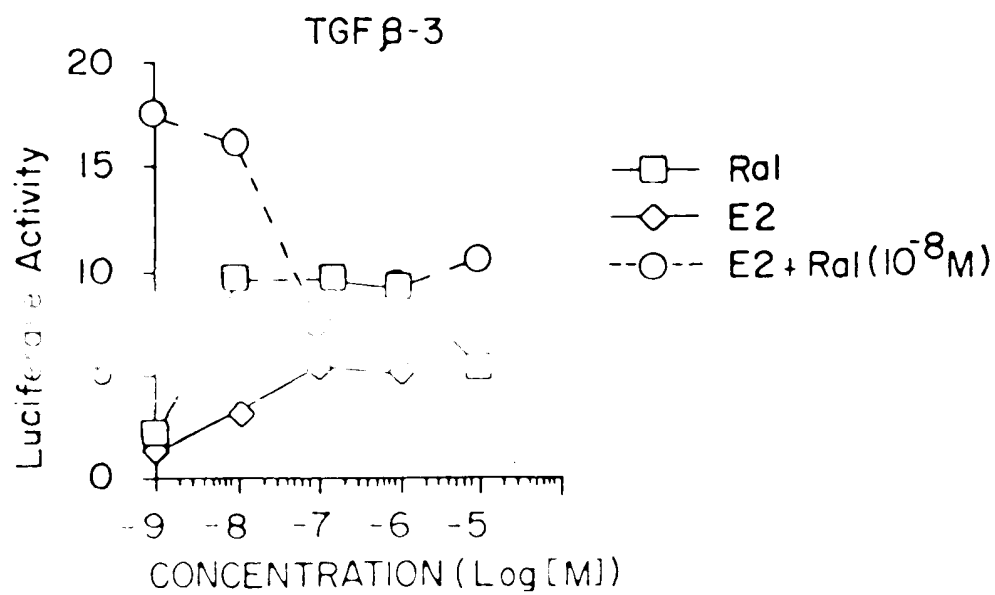


FIG. 6

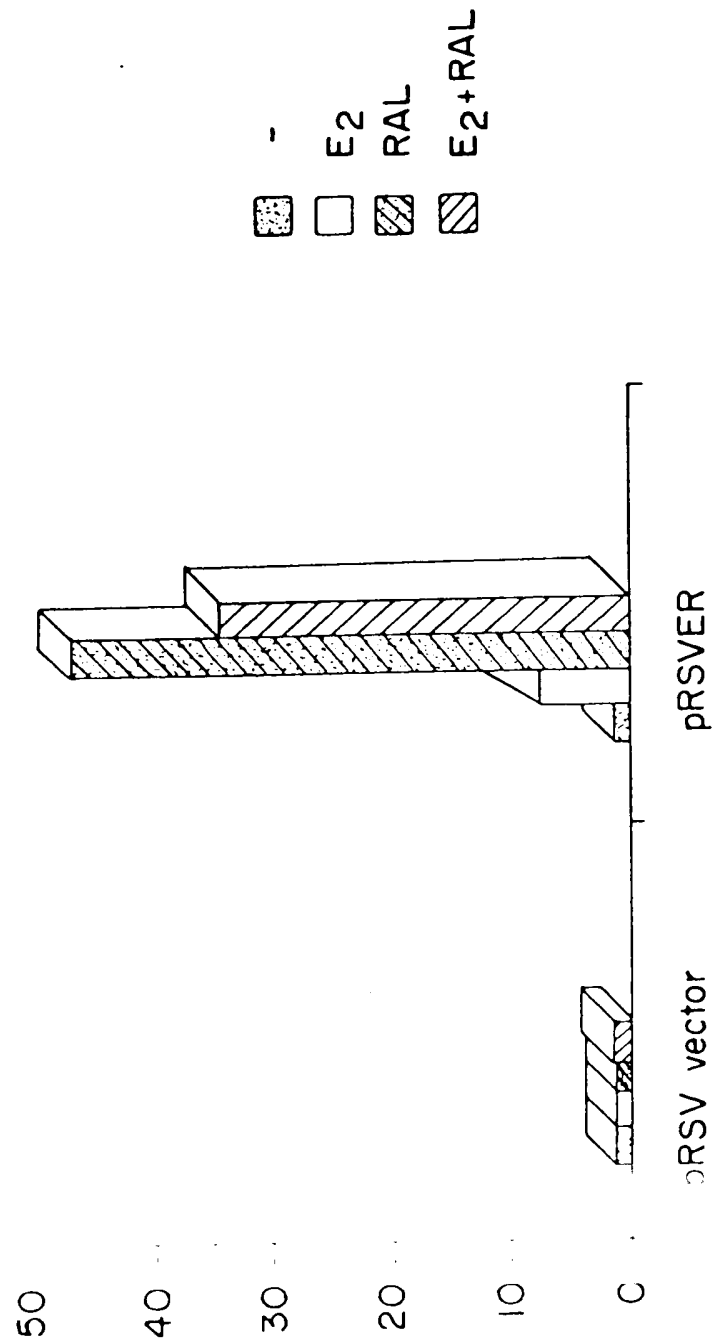


FIG. 7

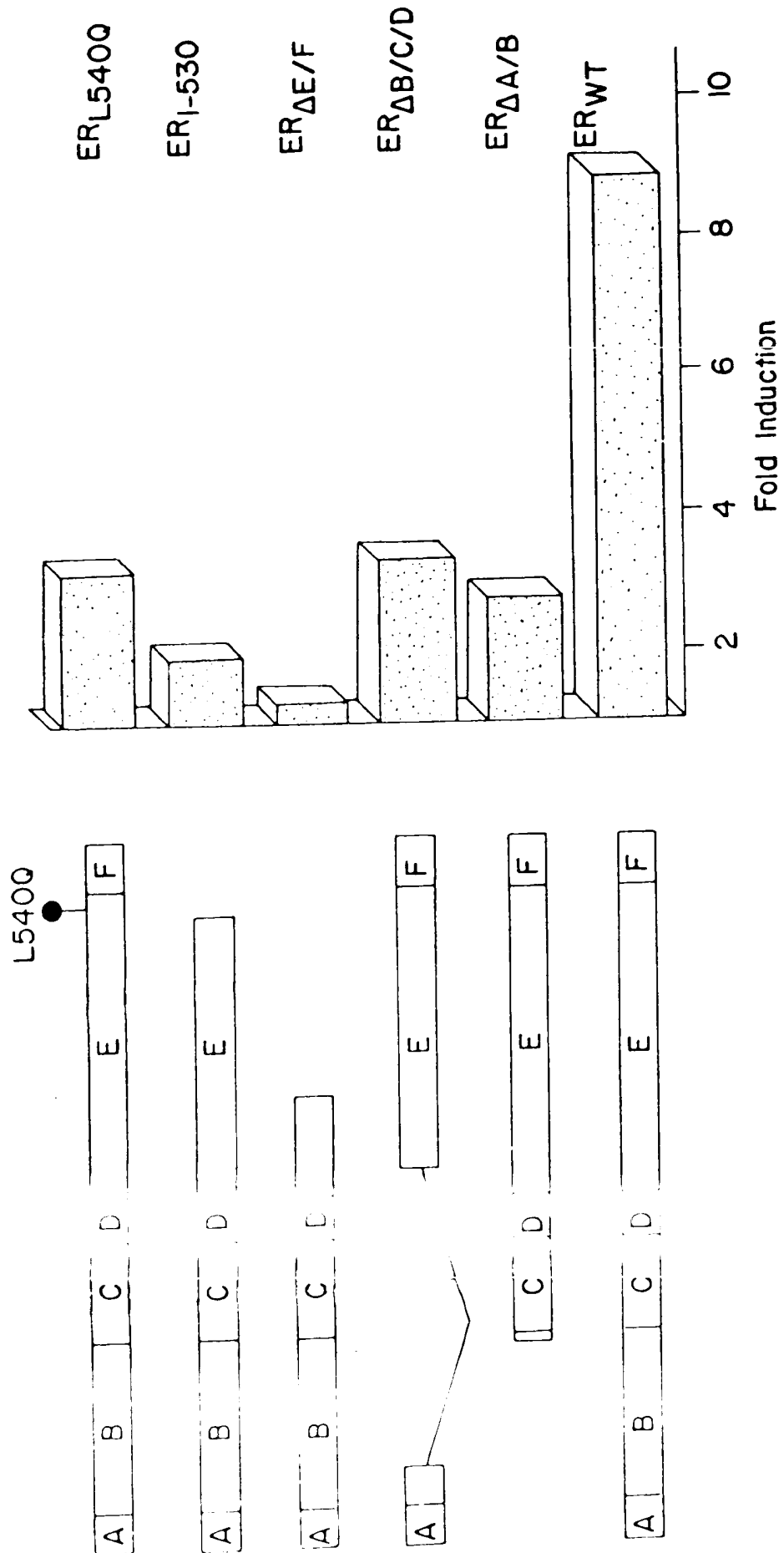


FIG. 8

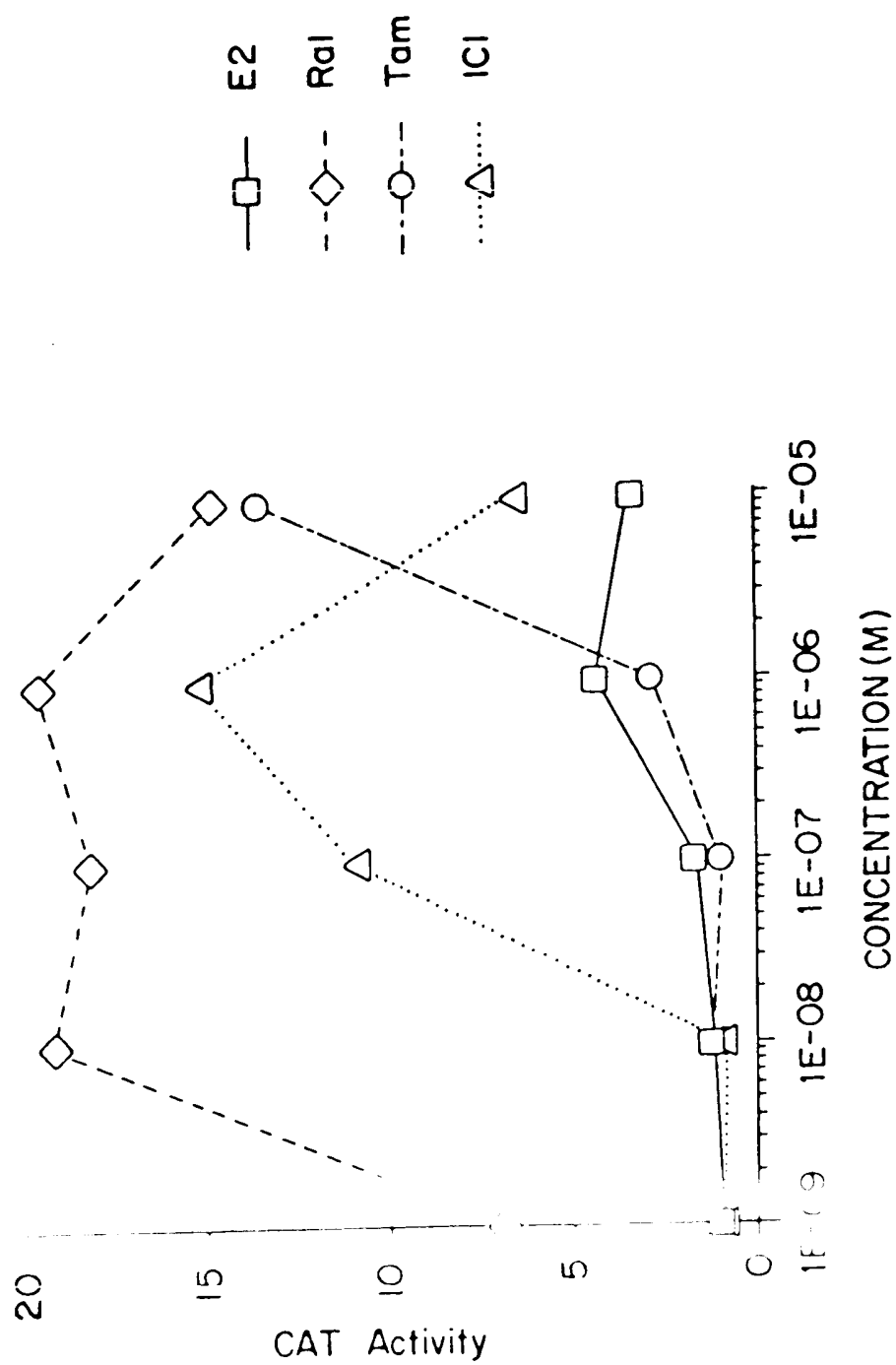


FIG. 9

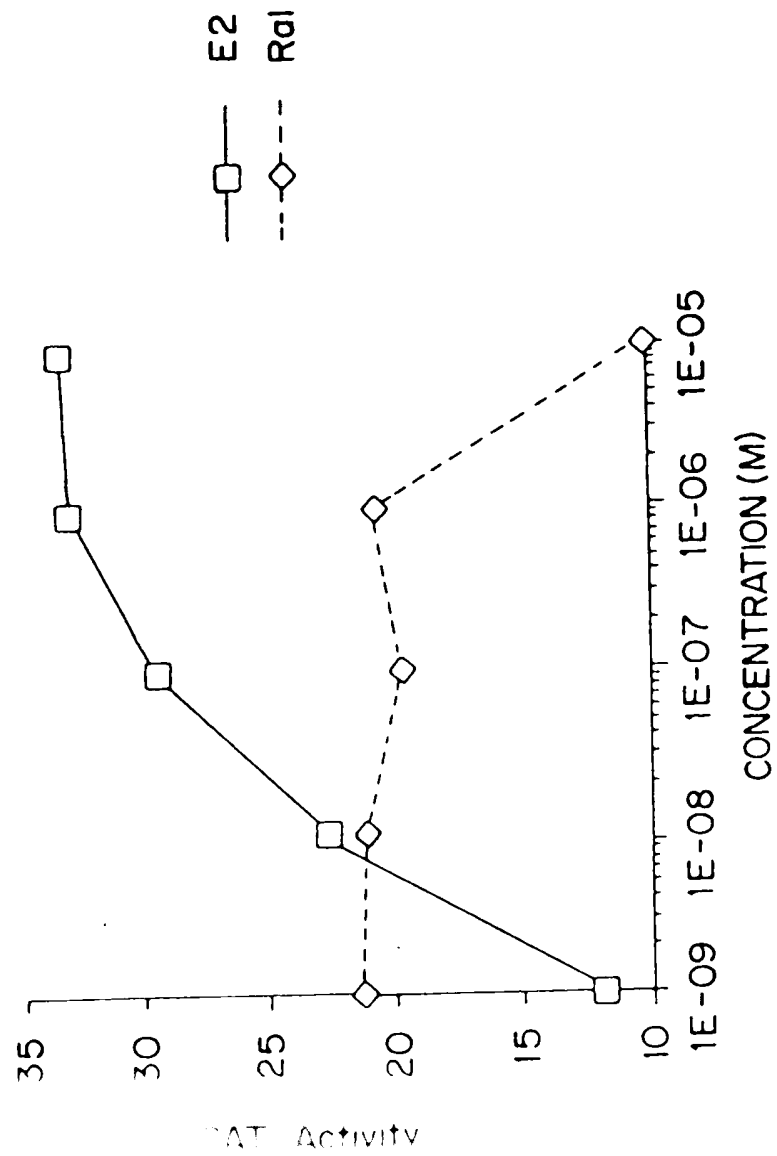


FIG.10

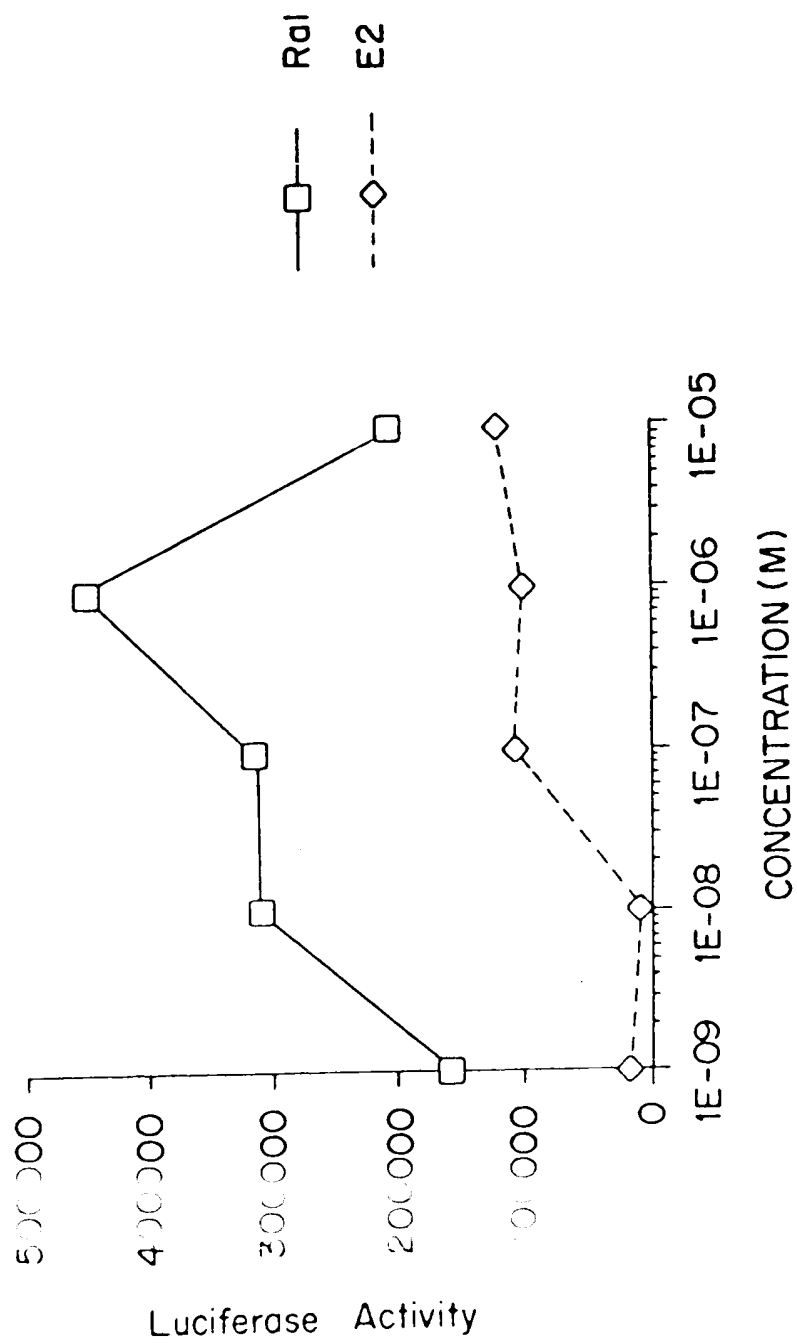
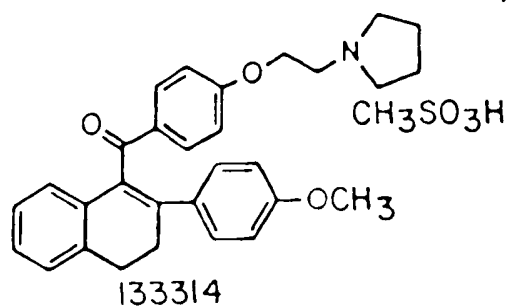
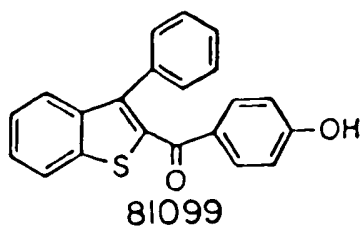
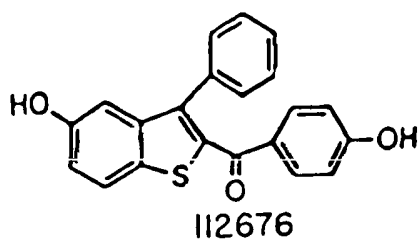


FIG. II

Estrogenic



Non-estrogenic

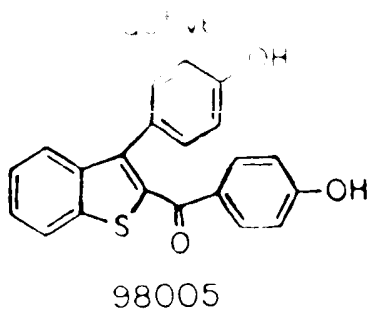
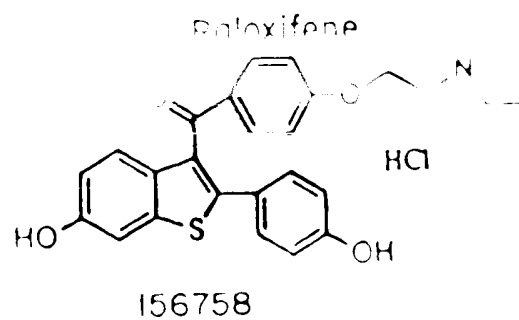
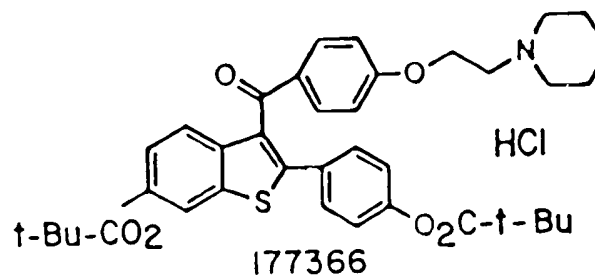
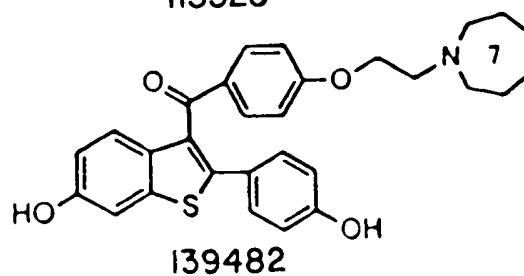
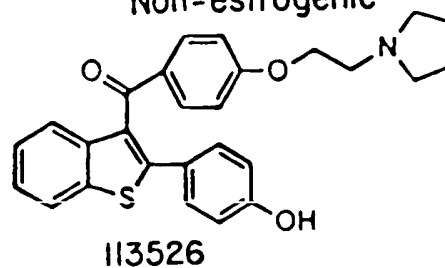


FIG. 12

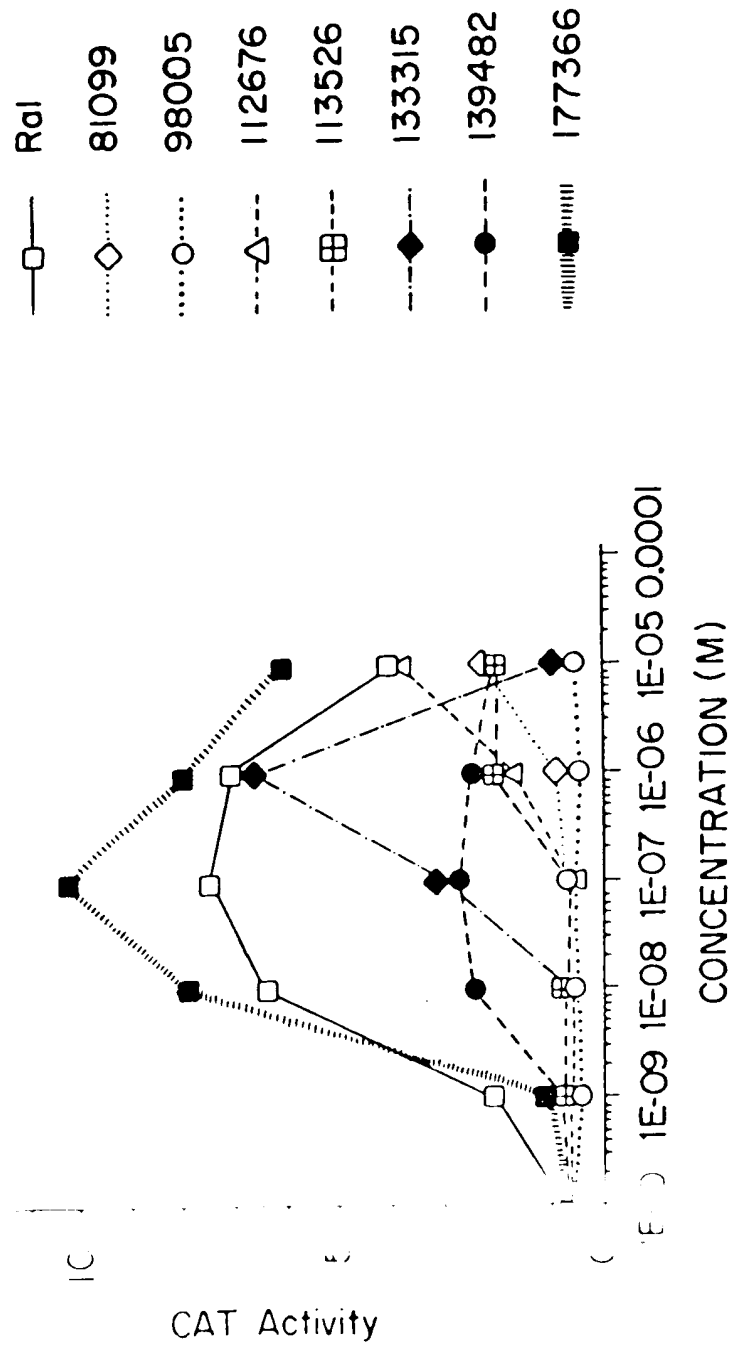


FIG. 13

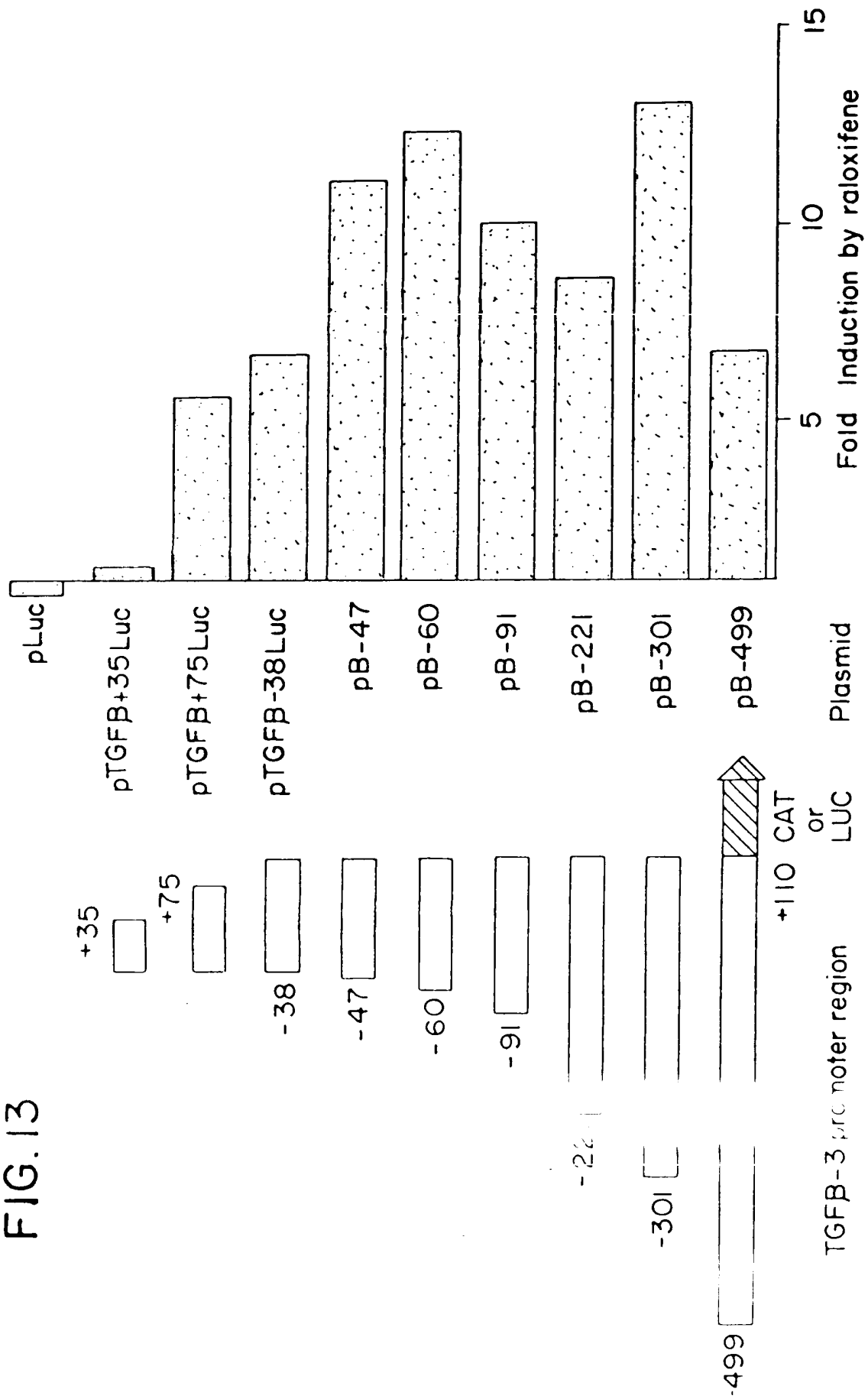


FIG. 14

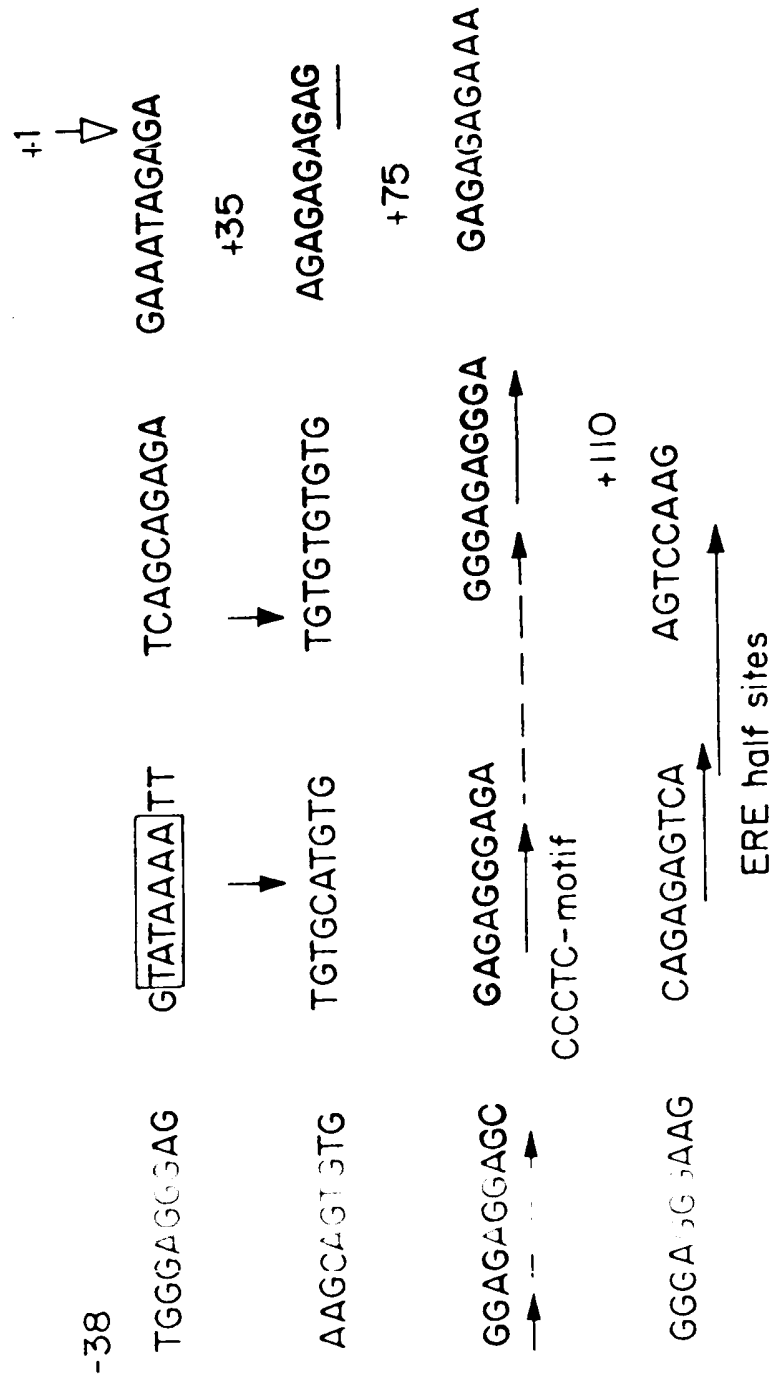


FIG.15

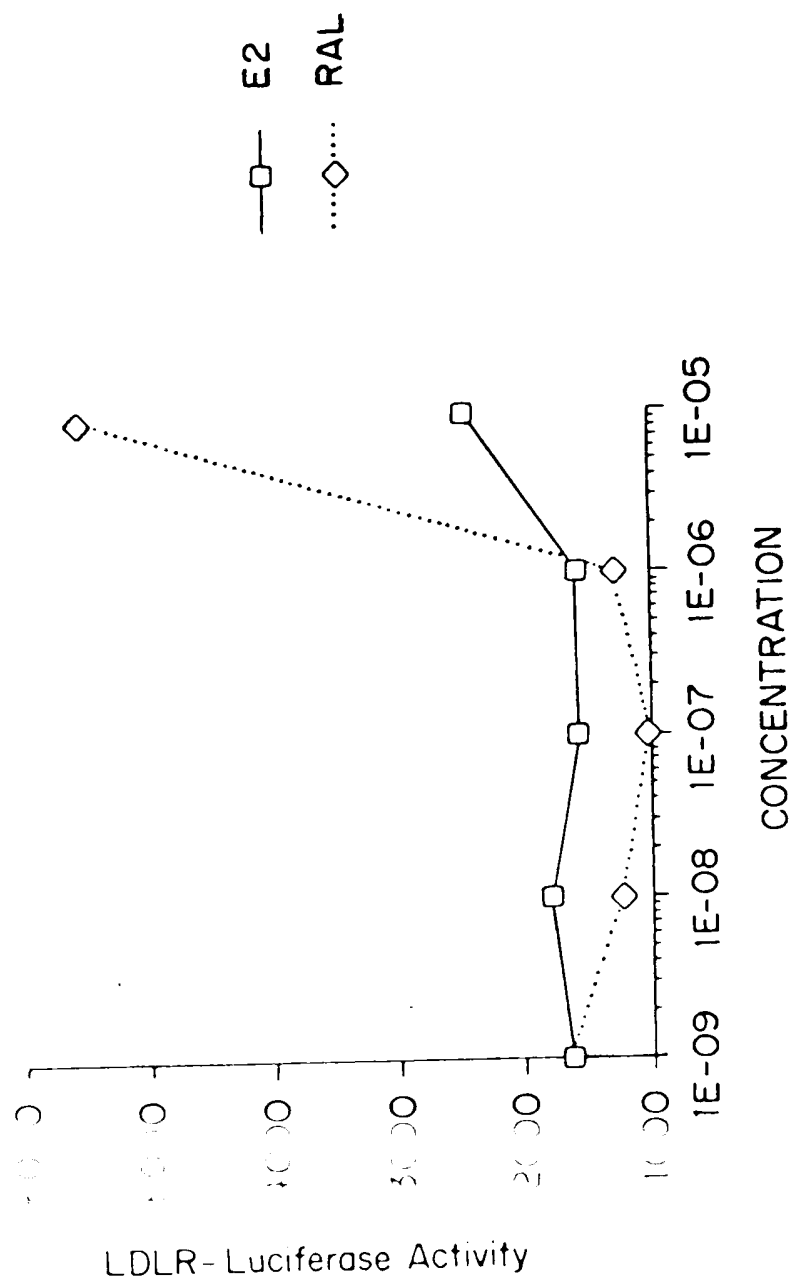


FIG.16

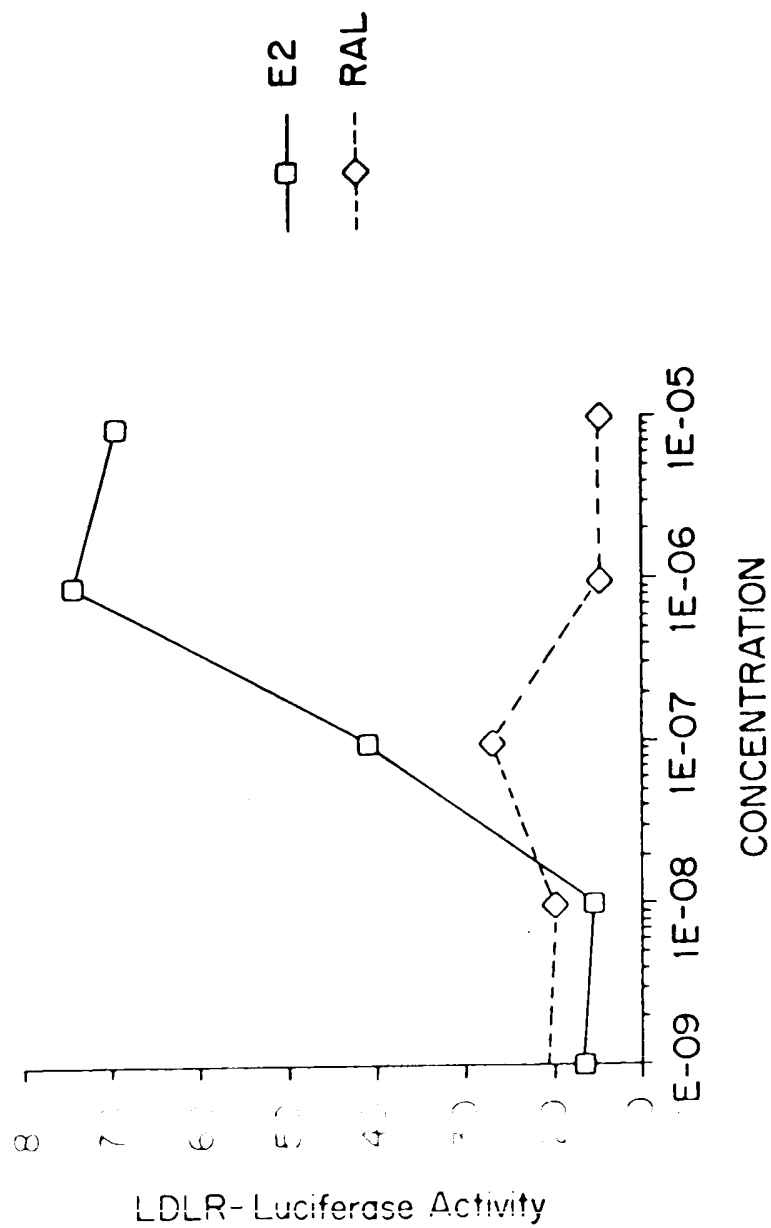


FIG. 17

